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(57) Abstract

A genetically engineered cell for inducing tolerance.

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GENETICALLY ENGINEERED SWINE CELLS

This application is a continuation-in-part of: USSN 08/266,427, filed June 27, 1994; USSN 08/266, 427, filed June 26, 1994; USSN 08/243,653 by Sykes and Sachs, filed May 16, 1994; USSN 08/220,371, filed March 29, 1994; USSN 08/212,228, filed March 14, 1994; USSN 08/163,912, filed December 7, 1993; USSN 08/150,739, filed November 10, 1993; USSN 08/126,122, filed on September 23, 1993; USSN 08/114,072, filed August 30, 1993; USSN 07/838,595, filed February 19, 1992; PCT/US94/05527, filed May 16, 1994; and PCT/US94/01616, filed February 14, 1994. All of the U.S. patent applications and PCT International Applications recited herein are hereby incorporated by reference.

Background of the Invention

The invention relates to the field of organ transplantation.

Technical advances in allogeneic organ transplantation and the availability of nonspecific immunosuppressive agents have revolutionized the field of organ transplantation. This progress has, however, resulted in a shortage of essential organs of suitable size and match.

The shortage of allograft-organs has led to an increased interest in xenogeneic transplantation. It was demonstrated more than twenty-five years ago that transplants from chimpanzee to man could provide long-term life-supporting function. However, the use of non-human primates as an organ source is of limited applicability. Many primate species are scarce and protected, and those that are more plentiful, such as the baboon, often do not grow to a size which allows the use of their organs in adults. Moreover, in some cultures, the use of primates as a source of organs is ethically unacceptable.

Some of these difficulties could be resolved by use of ungulate organs, especially pig organs. Pigs are domesticated, easy to breed, have large litters, and grow rapidly to the size which allow the use of their organs in the very largest human beings. In addition, pig and man have many anatomical and physiological similarities. However, transplantation of a pig organ into a human results in a vigorous rejection of the graft-organ.

Summary of the Invention

In general, the invention features, a genetically engineered swine cell, e.g., a cultured swine cell, e.g., a retrovirally transformed cultured swine cell, or a cell derived from a transgenic swine, which includes one or both of: a transgene encoding a graft-supporting protein, e.g., a primate, e.g., a human, hematopoietic peptide; or, a transgene which inhibits the expression or action of a gene product which is graft-antagonistic. Examples of transgenes which inhibit the expression or action of a gene product which is graft-antagonistic include: a transgene which encodes an anti-sense RNA which, directly

or indirectly, inhibits the expression or action of a recipient-derived graft-antagonistic protein, e.g., an anti-sense RNA which inhibits the expression of a donor-encoded receptor for a recipient-derived protein (and thereby inhibits the action of the recipient-derived protein; a transgene which is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein and which when inserted into the donor genome, e.g., by homologous recombination, results in an endogenous gene which is misexpressed or which is mutationally inactivated, by, e.g., the introduction of a mutation, e.g., a deletion, into an endogenous genomic copy of the gene which encodes the donor graft-antagonistic protein (e.g., the insertion of a transgene results in a knockout for a donor-derived receptor for a recipient-derived protein which is a graft antagonistic protein); a transgene which encodes an inhibitor of a donor- or recipient-derived graft-antagonistic protein, e.g., a competitive inhibitor or a protease or other molecule which specifically inhibits the activity of the graft antagonistic protein; and a transgene which encodes a dominant negative mutation in a gene product which is graft-antagonistic, e.g., a donor cell receptor for a host cytokine.

In preferred embodiments the transgene encoding a graft-supporting protein is: a recipient MHC gene, e.g., a primate, e.g., a non-human primate or a human, MHC gene.

In preferred embodiments the transgene is one which inhibits the expression or action of a donor MHC gene product (which gene product is graft-antagonistic).

In preferred embodiments the genetically engineered swine cell is a hematopoietic stem cell and the transgene encoding a graft-supporting protein is: a recipient MHC gene, e.g., a primate, e.g., a non-human primate or a human MHC gene.

In preferred embodiments the genetically engineered swine cell is a hematopoietic stem cell and the transgene is one which inhibits the expression or action of a donor MHC gene product (which gene product is graft-antagonistic).

In preferred embodiments the transgene encoding a graft-supporting protein and/or the transgene which inhibits the expression or action of a gene product which is graft-antagonistic is other than: an MHC gene; a swine MHC gene; a recipient MHC gene; a non-primate MHC gene; or a non-human MHC gene.

In preferred embodiments the genetically engineered swine cell is a hematopoietic stem cell and the transgene encoding a graft-supporting protein and/or the transgene which inhibits the expression or action of a gene product which is graft-antagonistic is other than: an MHC gene; a swine MHC gene; a recipient MHC gene; a non-primate, MHC gene; or a non-human MHC gene.

In preferred embodiments the transgene encodes a graft-supporting protein, e.g., a human growth factor or cytokine receptor, e.g., a growth factor or cytokine receptor involved in the regulation of hematopoiesis. Examples of growth factor or cytokine

receptor include the receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, and uteroferrin.

In other preferred embodiments the transgene encodes a graft-supporting protein, e.g., a human adhesion molecule, e.g., an adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells. Examples of human adhesion molecules include VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, and CD34.

In yet other preferred embodiments the transgene encodes a recipient or donor protein, e.g., a cytokine, which directly, or indirectly (e.g., by the stimulation or inhibition of the level of activity of a second cytokine), inhibits an immune response mounted by donor cells against the recipient, e.g., IL-10, IL-4, IL-2, or TGF-β.

In yet other preferred embodiments the transgene encodes a chimeric molecule, e.g., a chimeric lymphokine, e.g., PIXY123.

In yet other preferred embodiments the transgene encodes a graft-supporting protein, e.g., a recipient or donor cytokine, which directly, or indirectly (e.g., by the stimulation or inhibition of the level of activity of a second cytokine), inhibits an immune response mounted by recipient cells against donor tissue, e.g., IL-10, IL-4, IL-2, or TGF-β.

In yet other preferred embodiments the transgene inhibits the expression or action of a gene product which is graft-antagonistic, e.g., by decreasing the expression of the gene product. E.g., the transgene is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein, e.g., the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine, and which when inserted into the donor genome, e.g., by homologous recombination, results in an endogenous gene which is misexpressed or which is mutationally inactivated, by, e.g., the introduction of a mutation, e.g., a deletion, into an endogenous genomic copy of the gene which encodes the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine.

The transgene can be one which encodes an anti-sense RNA which, directly or indirectly, inhibits the expression or action of a recipient-derived graft-antagonistic protein, e.g., an anti-sense RNA which inhibits the expression of a donor-encoded B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine.

The transgene can be one which encodes a dominant negative mutation in a gene product which is graft-antagonistic, e.g., a donor cell receptor for a host cytokine or donor B-7 receptor, CD27 receptor, or LFA-3 receptor.

In yet other preferred embodiments the transgene includes a nucleic acid encoding a human peptide, e.g., a hematopoietic peptide, operably linked to: a promoter other than

the one it naturally occurs with; a swine promoter, e.g., a swine hematopoietic gene promoter; a viral promoter; or an inducible or developmentally regulated promoter.

In yet other preferred embodiments the genetically engineered swine cell is: a swine hematopoietic stem cell, e.g., a cord blood hematopoietic stem cell, a bone marrow hematopoietic stem cell, or a fetal or neonatal liver or spleen hematopoietic stem cell; derived from differentiated blood cells, e.g. a myeloid cell, such as a megakaryocytes, monocytes, granulocytes, or an eosinophils; an erythroid cell, such as a red blood cells, e.g. a lymphoid cell, such as B lymphocytes and T lymphocytes; derived from a pluripotent hematopoietic stem cell, e.g. a hematopoietic precursor, e.g. a burst-forming units-erythroid (BFU-E), a colony forming unit-erythroid (CFU-E), a colony forming unit-megakaryocyte (CFU-Meg), a colony forming unit-granulocyte-monocyte (CFU-GM), a colony forming unit-eosinophil (CFU-Eo), or a colony forming unit-granulocyte-erythrocyte-megakaryocyte-monocyte (CFU-GEMM); a swine cell other than a hematopoietic stem cell, or other blood cell; a swine thymic cell, e.g., a swine thymic stromal cell; a bone marrow stromal cell; a swine liver cell; a swine kidney cell; a swine epithelial cell; a swine hematopoietic progenitor cell; a swine muscle cell, e.g., a heart cell; or a dendritic cell or precursor thereof.

In yet other preferred embodiments the transgenic cell is: isolated or derived from cultured cells, e.g., a primary culture, e.g., a primary cell culture of hematopoietic stem cells; isolated or derived from a transgenic animal.

In yet other preferred embodiments: the transgenic swine cell is hemizygous for the transgene; the transgenic swine cell is heterozygous for the transgene; the transgenic swine cell is homozygous for the transgene (heterozygous transgenic swine can be bred to produce offspring that are homozygous for the transgene); the transgenic swine cell includes two or more transgenes.

In another aspect, the invention features, a transgene including a swine promoter, e.g., a swine hematopoietic gene promoter, or a heterologous inducible or developmentally regulated promoter, operably linked to either: a nucleic acid encoding a graft-supporting protein, e.g., a primate or a human graft-supporting protein, e.g., a primate, e.g., a human, hematopoietic peptide; or a nucleic acid which encodes or, a transgene which inhibits the expression or action of a gene product which is graft-antagonistic. Examples of transgenes which inhibit the expression or action of a gene product which is graft-antagonistic include: a transgene which encodes an anti-sense RNA which, directly or indirectly, inhibits the expression or action of a recipient-derived graft-antagonistic protein, e.g., an anti-sense RNA which inhibits the expression of a donor-encoded receptor for a recipient-derived protein (and thereby inhibits the action of the recipient-derived protein; a transgene which encodes an inhibitor of a donor- or recipient-derived graft-antagonistic protein, e.g., a competitive inhibitor or a protease or

other molecule which specifically inhibits the activity of the graft antagonistic protein; and a transgene which encodes a dominant negative mutation in a gene product which is graft-antagonistic, e.g., a donor cell receptor for a host cytokine.

In preferred embodiments the transgene encoding a graft-supporting protein is: a recipient MHC gene, e.g., a primate, e.g., a non-human primate or a human, MHC gene.

In preferred embodiments the transgene is one which inhibits the expression or action of a donor MHC gene product (which gene product is graft-antagonistic).

In preferred embodiments the nucleic acid encoding a graft-supporting protein and/or the nucleic acid which inhibits the expression or action of a gene product which is graft-antagonistic is other than: an MHC gene; a swine MHC gene; a recipient MHC gene; a non-primate MHC gene; or a non-human MHC gene.

In preferred embodiments the nucleic acid encodes a graft-supporting protein, e.g., a human growth factor or cytokine receptor, e.g., a growth factor or cytokine receptor involved in the regulation of hematopoiesis. Examples of growth factor or cytokine receptor include the receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, and uteroferrin.

In other preferred embodiments the nucleic acid encodes a graft-supporting protein, e.g., a human adhesion molecule, e.g., an adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells. Examples of human adhesion molecules include VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, and CD34.

In yet other preferred embodiments the nucleic acid encodes a recipient or donor protein, e.g., a cytokine, which directly, or indirectly (e.g., by the stimulation or inhibition of the level of activity of a second cytokine), inhibits an immune response mounted by donor cells against the recipient, e.g., IL-10, IL-4, IL-2, or TGF-β.

In yet other preferred embodiments the nucleic acid encodes a graft-supporting protein, e.g., a recipient or donor cytokine which directly, or indirectly (e.g., by the stimulation or inhibition of the level of activity of a second cytokine), inhibits an immune response mounted by recipient cells against donor tissue, e.g., IL-10, IL-4, IL-2, or TGF-β.

The transgene can be one which encodes an anti-sense RNA which, directly or indirectly, inhibits the expression or action of a recipient-derived graft-antagonistic protein, e.g., an anti-sense RNA which inhibits the expression of a donor-encoded B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine.

The transgene can be one which encodes a dominant negative mutation in a gene product which is graft-antagonistic, e.g., a donor cell receptor for a host cytokine or donor B-7 receptor, CD27 receptor, or LFA-3 receptor.

In yet other preferred embodiments the transgene encodes a chimeric molecule, e.g., a chimeric lymphokine, e.g., PIXY123.

In preferred embodiments, the transgene further includes transcriptional regulatory sequences, e.g. a tissue-specific promoter, e.g., a hematopoietic specific promoter, operably linked to the recombinant human gene sequence.

In another aspect, the invention features, a transgene which inhibits the action of a gene product which is graft-antagonistic, e.g., by decreasing the expression of the gene product. E.g., the transgene is a transgene which is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein and which when inserted into the donor genome, e.g., by homologous recombination, results in an endogenous gene which is misexpressed or which is mutationally inactivated, by, e.g., the introduction of a mutation, e.g., a deletion, into an endogenous genomic copy of the gene which encodes the donor graft-antagonistic protein (e.g., the insertion of a transgene results in a knockout for a donor-derived receptor for a recipient-derived protein which is a graft antagonistic protein).

In preferred embodiments the transgene is one which inhibits the expression or action of a donor MHC gene product (which gene product is graft-antagonistic).

In preferred embodiments the transgene which inhibits the action of a gene product which is graft-antagonistic is other than: an MHC gene; a swine MHC gene; a recipient MHC gene; a non-primate MHC gene; or a non-human MHC gene.

In yet other preferred embodiments the transgene inhibits the expression or action of a gene product which is graft-antagonistic, e.g., by decreasing the expression of the gene product. E.g., the transgene is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein, e.g., the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor , or a donor receptor for a host cytokine, and which when inserted into the donor genome, e.g., by homologous recombination, results in an endogenous gene which is misexpressed or which is mutationally inactivated, by, e.g., the introduction of a mutation, e.g., a deletion, into an endogenous genomic copy of the gene which encodes the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine.

In another aspect, the invention features, a transgenic swine having cells which include one or both of: a transgene encoding a graft-supporting protein, e.g., a primate or a human graft-supporting protein, e.g., a primate, e.g., a human, protein, preferably a hematopoietic peptide; or, a transgene which inhibits the expression or action of a gene product which is graft-antagonistic. Examples of transgenes which inhibit the expression or action of a gene product which is graft-antagonistic include: a transgene which encodes an anti-sense RNA which, directly or indirectly, inhibits the expression or action of a recipient-derived graft-antagonistic protein, e.g., an anti-sense RNA which inhibits the

expression of a donor-encoded receptor for a recipient-derived protein (and thereby inhibits the action of the recipient-derived protein; a transgene which is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein and which when inserted into the donor genome, e.g., by homologous recombination, results in an endogenous gene which is misexpressed or which is mutationally inactivated, by, e.g., the introduction of a mutation, e.g., a deletion, into an endogenous genomic copy of the gene which encodes the donor graft-antagonistic protein (e.g., the insertion of a transgene results in a knockout for a donor-derived receptor for a recipient-derived protein which is a graft antagonistic protein); a transgene which encodes an inhibitor of a donor- or recipient-derived graft-antagonistic protein, e.g., a competitive inhibitor or a protease or other molecule which specifically inhibits the activity of the graft antagonistic protein; and a transgene which encodes a dominant negative mutation in a gene product which is graft-antagonistic, e.g., a donor cell receptor for a host cytokine.

In preferred embodiments the transgene encoding a graft-supporting protein is: a recipient MHC gene, e.g., a primate, e.g., a non-human primate or a human, MHC gene.

In preferred embodiments the transgene is one which inhibits the expression or action of a donor MHC gene product (which gene product is graft-antagonistic).

In preferred embodiments the transgene encoding a graft-supporting protein and/or the transgene which inhibits the expression or action of a gene product which is graft-antagonistic is other than: an MHC gene; a swine MHC gene; a recipient MHC gene; a non-primate MHC gene; or a non-human MHC gene.

In preferred embodiments the transgene encodes a graft-supporting protein, e.g., a human growth factor or cytokine receptor, e.g., a growth factor or cytokine receptor involved in the regulation of hematopoiesis. Examples of growth factor or cytokine receptor include the receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, and uteroferrin.

In other preferred embodiments the transgene encodes a graft-supporting protein, e.g., a human adhesion molecule, e.g., an adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells. Examples of human adhesion molecules include VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, and CD34.

In yet other preferred embodiments the transgene encodes a recipient or donor protein, e.g., a cytokine which directly, or indirectly (e.g., by the stimulation or inhibition of the level of activity of a second cytokine), inhibits an immune response mounted by donor cells against the recipient, e.g., IL-10, IL-4, IL-2, or TGF-β.

In yet other preferred embodiments the transgene encodes a graft-supporting protein, e.g., a recipient or donor cytokine which directly, or indirectly (e.g., by the stimulation or inhibition of the level of activity of a second cytokine) inhibits an immune

response mounted by recipient cells against donor tissue, e.g., IL-10, IL-4, IL-2, or TGF-β.

In yet other preferred embodiments the transgene encodes a chimeric molecule, e.g., a chimeric lymphokine, e.g., PIXY123.

In yet other preferred embodiments the transgene inhibits the expression or action of a gene product which is graft-antagonistic, e.g., by decreasing the expression of the gene product. E.g., the transgene is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein, e.g., the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine, and which when inserted into the donor genome, e.g., by homologous recombination, results in an endogenous gene which is misexpressed or which is mutationally inactivated, by, e.g., the introduction of a mutation, e.g., a deletion, into an endogenous genomic copy of the gene which encodes the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine.

The transgene can be one which encodes an anti-sense RNA which, directly or indirectly, inhibits the expression or action of a recipient-derived graft-antagonistic protein, e.g., an anti-sense RNA which inhibits the expression of a donor-encoded B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine.

The transgene can be one which encodes a dominant negative mutation in a gene product which is graft-antagonistic, e.g., a donor cell receptor for a host cytokine or donor B-7 receptor, CD27 receptor, or LFA-3 receptor.

In yet other preferred embodiments the transgene includes a nucleic acid encoding a graft-supporting protein, e.g., a primate or human graft-supporting protein, e.g., a primate or human hematopoietic peptide or a transgene which inhibits the action of a gene product which is a graft-antagonistic, e.g., a gene product which is the donor receptor for a recipient protein which is a graft antagonistic protein operably linked to: a promoter other than the one it naturally occurs with; a swine promoter, e.g., a swine hematopoietic gene promoter; a viral promoter; an inducible promoter; or a developmentally regulated promoter.

In yet other preferred embodiments: the transgenic swine cell is hemizygous for the transgene; the transgenic swine cell is hemizygous for the transgene; the transgenic swine is heterozygous for the transgene; the transgenic swine is homozygous for the transgene (heterozygous transgenic swine can be bred to produce offspring that are homozygous for the transgene); the transgenic swine includes two or more transgenes.

Transgenic swine (or swine cells) of the invention can be used as a source for "humanized" hematopoietic cells for xenogeneic grafting into human subjects. Transgenic swine or swine cells of the invention can also be used to measure and/or

identify agonists or antagonists of a human growth factor, cytokine, or other molecule involved in hematopoietic regulation.

Transgenic swine cells of the invention derived, e.g., from retrovirally transformed cultured cells, or from a transgenic animal, can be used to induce immunologic tolerance in a recipient animal to a graft from a donor swine. For example, cells of the invention can be combined with methods of inducing tolerance described in USSN 08/126,122, filed September 23, 1993.

The invention provides for the implantation of swine donor cells which have been engineered to increase desirable interactions between the donor cells and molecules and cells of a recipient, e.g., to promote the engraftment or function of the donor stem cells in the recipient environment. (Generally, stem cells are implanted to induce tolerance to (or otherwise promote acceptance of) donor graft cells.) The invention also provides for the implantation of donor cells which have been engineered to minimize unwanted interactions between the donor cells and molecules and cells of the recipient which, e.g., promote the rejection of donor graft cells or which inhibit the function of the donor graft cells. The invention provides for transplantation methods wherein either, or both, the stem cells and the graft cells are so engineered. Engineered alterations which increase desired interactions between donor stem cells and the recipient may, in some cases, be undesirable in the cells of the graft. Likewise engineered alterations which decrease unwanted interactions between the donor graft cells and the recipient may, in some cases, be undesirable in the donor stem cells. Thus, the invention includes methods in which the donor stem cells and the donor graft differ in that one has an engineered alteration which the other lacks. E.g., in some applications the donor stem cells will have a transgene not present in the graft cells and the donor graft cells will have a transgene not present in the donor stem cells.

Accordingly, in another aspect, the invention features, a method of inducing tolerance in a recipient mammal, e.g., a primate, e.g., a human, to graft cells (or otherwise promoting the acceptance by a recipient mammal of graft cells) from a donor mammal, e.g., a miniature swine, including:

introducing into the recipient, donor hematopoietic stem cells, and

introducing into the recipient, donor graft cells,

provided that at least one of the following conditions is met: (1) the donor stem cells have been genetically engineered to promote a desirable interaction between the donor stem cells and cells or molecules of the recipient; (2) the donor stem cells have been genetically engineered to inhibit an unwanted interaction between cells or molecules of the recipient and the donor stem cells; (3) the donor graft cells have been genetically engineered to promote a desirable interaction between the donor graft (and/or stem) cells and cells or molecules of the recipient; or (4) the donor graft cells have been genetically

engineered to inhibit an unwanted interaction between cells or molecules of the recipient and the donor graft (and/or stem) cells.

In preferred embodiments: if the genetically engineered alteration in (1) or (2) is the insertion of an MHC gene, e.g., a swine MHC, a donor MHC gene, a recipient MHC gene, a non-primate MHC gene, or a non-human MHC gene, then one or both of, donor cells which are genetically altered by other than the insertion of an MHC gene, or, genetically altered cells other than hematopoietic stem cells, are also introduced into the recipient.

In preferred embodiments: if a cell having a transgene which is an MHC gene, e.g., a swine MHC, a donor MHC gene, a recipient MHC gene, a non-primate MHC gene, or a non-human MHC gene, is administered to the recipient, then a second cell, which has a transgene other than an MHC gene, e.g., a swine MHC, a donor MHC gene, or a recipient MHC gene, a non-primate MHC gene, or a non-human MHC gene, is also administered to the recipient.

In preferred embodiments: genetically engineered refers to the inclusion of a transgene; the donor stem cells have a genetically engineered alteration, e.g., a transgene, which the donor graft cells lack; the donor graft cells have a genetically engineered alteration, e.g., a transgene, which the donor stem cells lack; the donor stem cells have a first genetically engineered alteration, e.g., a first transgene, which increases an interaction between the stem cells and molecules or cells of the recipient and the donor graft cells have a second genetically engineered alteration, e.g., a second transgene, which decreases an interaction between the donor graft cells and molecules or cells of the recipient.

In preferred embodiments: the donor stem cells include a transgene which encodes a graft-supporting protein, e.g., a hematopoietic peptide and the donor graft cells do not include the transgene of the donor stem cells.

In other preferred embodiments: the donor graft cells include a transgene which inhibits the action of a gene product which is a graft-antagonistic, e.g., a gene product which is the receptor for a recipient protein which is a graft antagonistic protein and the donor stem cells do not include the transgene.

In yet other preferred embodiments: the donor stem cells include a transgene which encodes a graft-supporting protein, e.g., a human growth factor or cytokine receptor, e.g., a growth factor or cytokine receptor involved in the regulation of hematopoiesis. Examples of growth factor or cytokine receptor include the receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, and uteroferrin.

In yet other preferred embodiments: the donor stem cells include a transgene encodes a graft-supporting protein, e.g., a human adhesion molecule, e.g., an adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells. Examples

of human adhesion molecules include VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, and CD34.

In yet other preferred embodiments: the donor stem cells, the donor graft cells, or both, include a transgene which encodes a recipient or donor protein, a cytokine which directly, or indirectly (e.g., by the stimulation or inhibition of the level of activity of a second cytokine) inhibits an immune response mounted by donor cells against the recipient, e.g., IL-10, IL-4, IL-2, or TGF-β.

In yet other preferred embodiments: the donor stem cells, the donor graft cells, or both, include a transgene which encodes a graft-supporting protein, e.g., a recipient or donor cytokine which directly, or indirectly (e.g., by the stimulation or inhibition of the level of activity of a second cytokine) inhibits an immune response mounted by recipient cells against donor tissue, e.g., IL-10, IL-4, IL-2 or TGF-β.

In yet other preferred embodiments: the donor stem cells, the donor graft cells, or both, include a transgene which inhibits the expression or action of a gene product which is graft-antagonistic. Examples of transgenes which inhibit the expression or action of a gene product which is graft-antagonistic include: a transgene which encodes an anti-sense RNA which, directly or indirectly, inhibits the expression or action of a recipient-derived graft-antagonistic protein, e.g., an anti-sense RNA which inhibits the expression of a donor-encoded receptor for a recipient-derived protein (and thereby inhibits the action of the recipient-derived protein; a transgene which is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein and which when inserted into the donor genome, e.g., by homologous recombination, results in an endogenous gene which is misexpressed or which is mutationally inactivated, by, e.g., the introduction of a mutation, e.g., a deletion, into an endogenous genomic copy of the gene which encodes the donor graft-antagonistic protein (e.g., the insertion of a transgene results in a knockout for a donor-derived receptor for a recipient-derived protein which is a graft antagonistic protein); a transgene which encodes an inhibitor of a donor- or recipient-derived graft-antagonistic protein, e.g., a competitive inhibitor or a protease or other molecule which specifically inhibits the activity of the graft antagonistic protein; and a transgene which encodes a dominant negative mutation in a gene product which is graft-antagonistic, e.g., a donor cell receptor for a host cytokine.

In yet other preferred embodiments the transgene inhibits the expression or action of a gene product which is graft-antagonistic, e.g., by decreasing the expression of the gene product. E.g., the transgene is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein, e.g., the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine, and which when inserted into the donor genome, e.g., by homologous recombination, results in an endogenous gene which is misexpressed or which is mutationally inactivated, by, e.g., the

introduction of a mutation, e.g., a deletion, into an endogenous genomic copy of the gene which encodes the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine.

The transgene can be one which encodes an anti-sense RNA which, directly or indirectly, inhibits the expression or action of a recipient-derived graft-antagonistic protein, e.g., an anti-sense RNA which inhibits the expression of a donor-encoded B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine.

The transgene can be one which encodes a dominant negative mutation in a gene product which is graft-antagonistic, e.g., a donor cell receptor for a host cytokine or donor B-7 receptor, CD27 receptor, or LFA-3 receptor.

In yet other preferred embodiments, the donor stem cells, the donor graft, or both, include a transgene which encodes a chimeric molecule, e.g., a chimeric lymphokine, e.g., PIXY123.

In yet other preferred embodiments: the donor mammal and the recipient mammal are of different species, e.g., they are discordant primates, e.g., a human recipient and a nonhuman donor; the recipient is a primate, e.g., a human; the donor is a swine e.g., a miniature swine.

In yet other preferred embodiments: the donor mammal and the recipient mammal are of the same species, e.g., both are primates, e.g., humans; the recipient and donor are of the same species and the donor stem cells include a transgene, introduced e.g., by retroviral transformation of cultured donor stem cells, and the cells of the donor graft do not include a transgene.

In yet other preferred embodiments the donor of the stem cells and the donor of the graft are both miniature swine and: the stem cell donor is from a strain which has been genetically engineered to express a graft supporting protein; the graft donor is a strain has been genetically engineered to have decreased expression for a protein which is antagonistic to graft acceptance or function; the graft donor and the stem cell donor are inbred; the graft donor and the stem cell donor are MHC identical.

In yet other preferred embodiments the transgene includes a nucleic acid operably linked to; a promoter other than the one it naturally occurs with; a swine promoter, e.g., a swine hematopoietic gene promoter; a viral promoter; or an inducible or developmentally regulated promoter.

In yet other preferred embodiments the genetically engineered swine stem cell is: isolated or derived from cultured cells, e.g., a primary culture, e.g., a primary culture of hematopoietic stem cells; isolated or derived from a transgenic animal; isolated or derived from cord blood; obtained from an individual animal from which the graft cells are obtained; obtained from an individual animal which is syngeneic with the individual animal from which the graft cells are obtained; obtained from an individual animal which

is MHC matched, and preferably identical, with the individual animal from which the graft cells are obtained; a cord blood, a bone marrow hematopoietic stem cell, or a fetal or neonatal liver or spleen cell hematopoietic stem cell.

In preferred embodiments: the donor graft cells are other than a hematopoietic stem cells, or other blood cells; the donor graft cells are swine thymic cells, e.g., swine thymic stromal cells; the donor graft cells are bone marrow stromal cells; the donor graft cells are swine liver cells; the donor graft cells are swine kidney cells; the donor graft cells are swine epithelial cells; the donor graft cells are swine muscle cells, e.g., heart cells; the donor graft cells are swine neuronal cells; the graft cells include an organ, e.g., a kidney, a liver, or a heart; the donor graft cells include dendritic cells or their precursors.

Other preferred embodiments include: the step of introducing into the recipient, donor species-specific stromal tissue, preferably hematopoietic stromal tissue, e.g., fetal liver or thymus. In preferred embodiments: the stromal tissue is introduced simultaneously with, or prior to, the hematopoietic stem cells. In preferred embodiment: the stromal tissue has been: genetically engineered to promote a desirable interaction between the stromal cells and cells or molecules of the recipient; genetically engineered to inhibit an unwanted interaction between cells or molecules of the recipient and the stromal cells.

Other preferred embodiments include those in which: the transgenic stem cells are administered to the recipient prior to or simultaneous with transplantation of graft cells; the hematopoietic stem cells home to a site in the recipient; the stem cells are administered by intravenous injection.

Other preferred embodiments include (preferably prior to administering the stem cells): inactivating the natural killer cells of the recipient mammal, e.g., by introducing into the recipient mammal an antibody capable of binding to natural killer cells of the recipient; inactivating the T cells of the recipient mammal, e.g., by introducing into the recipient an antibody capable of binding to T cells of the recipient.

Other preferred embodiments include: the step of creating hematopoietic space, e.g., by one or more of, irradiating the recipient with low dose, e.g., between about 100 and 400 rads, whole body irradiation, administering a myleosuppressive drug to the recipient, or administering anti-class I antibodies to the recipient, to deplete or partially deplete the bone marrow of the recipient; the method includes the a step which creates hematopoietic space and the step is performed prior to introducing the transgenic cells into the recipient.

Other preferred embodiments include inactivating thymic T cells by one or more of: (preferably prior to hematopoietic stem cell transplantation) irradiating the recipient mammal with, e.g., about 700 rads of thymic irradiation; administering one, or preferably two or more, doses of an anti-T cell antibody; or administering to the recipient a short

course of an immunosuppressant as described in USSN 08/220,371, filed March 29, 1994.

Other preferred embodiments include: the step of depleting or otherwise inactivating natural antibodies in the blood of the recipient mammal, e.g., by hemoperfusing an organ, e.g., a liver or a kidney, obtained from a pig or administering a drug, e.g., deoxyspergualin (DSG) which inactivates or depletes natural antibodies; the method includes a step which depletes or otherwise inactivates natural antibodies in the blood of the recipient and the step is performed prior to hematopoietic stem cell transplantation.

Transgenic swine cells of the invention derived, e.g., from retrovirally transformed cultured cells, or from a transgenic animal, can be used in any method calling for the engraftment of swine hematopoietic cells in a xenogeneic environment. For example, cells of the invention can be combined with: methods which induce tolerance or otherwise promote the acceptance of a graft by administration of a short course of cyclosporine or similar agents, e.g., the methods described in USSN 08/220,371, filed March 29, 1994; methods which use the implantation of a xenogeneic thymic graft to induce tolerance, e.g., the methods described in USSN 08/163, 912 filed on December 7, 1993; methods of increasing the level of the activity of a tolerance promoting or GVHD inhibiting cytokine or decreasing the level of activity of an a tolerance inhibiting or GVHD promoting cytokine, e.g., the methods described in USSN 08/114,072, filed August 30, 1993; methods of using cord blood cells to induce tolerance, e.g., the methods described in USSN 08/150,739, filed November 10, 1993; the methods of USSN 08/126,122, filed September 23, 1993; and the methods for inducing tolerance disclosed in Sykes and Sachs, PCT/US94/01616, filed February 14, 1994.

In another aspect, the invention features, a method of promoting the engraftment and or repopulation of the bone marrow of a xenogeneic recipient, e.g., a primate, e.g., a human, by donor swine hematopoietic stem cells and thereby inducing mixed chimerism in the xenogeneic recipient. The method includes: providing a genetically engineered swine cell (which may or may not be a hematopoietic stem cell) which has been genetically engineered to promote a desirable interaction between donor stem cells and cells or molecules of the recipient or which have been genetically engineered to inhibit an unwanted interaction between the recipient and donor stem cells; and, implanting the genetically engineered swine cell in the recipient, provided that, if the genetically engineered swine cell is not a swine hematopoietic stem cell, a swine hematopoietic stem *cell is also implanted in the recipient.*

In preferred embodiments: the genetically engineered alteration is other than the insertion of an MHC gene, e.g., a swine MHC, a donor MHC gene, a recipient MHC gene, a non-primate MHC gene, or a non-human MHC gene.

In preferred embodiments: genetically engineered refers to the inclusion of a transgene.

In other preferred embodiments: the genetically engineered swine cells include a transgene which encodes a graft-supporting protein, e.g., a human growth factor or cytokine receptor, e.g., a growth factor or cytokine receptor involved in the regulation of hematopoiesis. Examples of growth factor or cytokine receptor include the receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, and uteroferrin.

In yet other preferred embodiments: the genetically engineered swine cells include a transgene encodes a graft-supporting protein, e.g., a human adhesion molecule, e.g., an adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells. Examples of human adhesion molecules include VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, and CD34.

In yet other preferred embodiments the genetically engineered swine cells, include a transgene which inhibits the expression or action of a gene product which is graft-antagonistic. Examples of transgenes which inhibit the expression or action of a gene product which is graft-antagonistic include: a transgene which encodes an anti-sense RNA which, directly or indirectly, inhibits the expression or action of a recipient-derived graft-antagonistic protein, e.g., an anti-sense RNA which inhibits the expression of a donor-encoded receptor for a recipient-derived protein (and thereby inhibits the action of the recipient-derived protein; a transgene which is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein and which when inserted into the donor genome, e.g., by homologous recombination, results in an endogenous gene which is misexpressed or which is mutationally inactivated, by, e.g., the introduction of a mutation, e.g., a deletion, into an endogenous genomic copy of the gene which encodes the donor graft-antagonistic protein (e.g., the insertion of a transgene results in a knockout for a donor-derived receptor for a recipient-derived protein which is a graft antagonistic protein); a transgene which encodes an inhibitor of a donor- or recipient-derived graft-antagonistic protein, e.g., a competitive inhibitor or a protease or other molecule which specifically inhibits the activity of the graft antagonistic protein; and a transgene which encodes a dominant negative mutation in a gene product which is graft-antagonistic, e.g., a donor cell receptor for a host cytokine.

In yet other preferred embodiments, the genetically engineered swine cells include a transgene which inhibits the action of, e.g., by decreasing the expression of, a gene product which is a graft-antagonistic. E.g., the transgene is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein, e.g., the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine, and which when inserted into the donor genome, e.g., by homologous recombination, results

in an endogenous gene which is misexpressed or which is mutationally inactivated, by, e.g., the introduction of a mutation, e.g., a deletion, into an endogenous genomic copy of the gene which encodes the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine.

The transgene can be one which encodes an anti-sense RNA which, directly or indirectly, inhibits the expression or action of a recipient-derived graft-antagonistic protein, e.g., an anti-sense RNA which inhibits the expression of a donor-encoded B-7 receptor, CD27 receptor, or LFA-3 receptor, or a donor receptor for a host cytokine.

The transgene can be one which encodes a dominant negative mutation in a gene product which is graft-antagonistic, e.g., a donor cell receptor for a host cytokine or donor B-7 receptor, CD27 receptor, or LFA-3 receptor.

In yet other preferred embodiments: the genetically engineered swine cells, include a transgene which encodes a recipient or donor protein, a cytokine which directly, or indirectly (e.g., by the stimulation or inhibition of the level of activity of a second cytokine) inhibits an immune response mounted by donor cells against the recipient, e.g., IL-10, IL-4, or TGF-β.

In yet other preferred embodiments: the genetically engineered swine cells, include a transgene which encodes a graft-supporting protein, e.g., a recipient or donor cytokine which directly, or indirectly (e.g., by the stimulation or inhibition of the level of activity of a second cytokine) inhibits an immune response mounted by recipient cells against donor tissue, e.g., IL-10, IL-4, or TGF-β.

In yet other preferred embodiments the genetically engineered swine cells include a transgene which encodes a chimeric molecule, e.g., a chimeric lymphokine, e.g., PIXY123.

In yet other preferred embodiments: the genetically engineered swine cell is isolated or derived from a cultured cell, e.g., a primary culture, e.g., a primary culture of hematopoietic stem cells; the genetically engineered swine cell is isolated or derived from a transgenic animal; if the genetically engineered swine cell is not a stem cell, then the genetically engineered swine cell and the stem cell which is also administered are obtained from the same animal, from animals which are (except for the alteration of the invention) syngeneic, or from animals which are MHC matched, and preferably MHC identical; the genetically engineered swine cell is a cord blood cell, a bone marrow hematopoietic stem cell, or a fetal or neonatal liver or spleen cell hematopoietic stem cell.

In preferred embodiments: the genetically engineered swine cells are other than hematopoietic stem cells, or other blood cells; the genetically engineered swine cells are thymic cells, e.g., swine thymic stromal cells; the genetically engineered swine cells are bone marrow stromal cells; the genetically engineered swine cells are swine liver cells; the genetically engineered swine cells are swine kidney cells; the genetically engineered

swine cells are swine epithelial cells; the genetically engineered swine cells are swine muscle cells, e.g., heart cells; the genetically engineered swine cells are swine neuronal cells; the genetically engineered swine cells are swine dentritic cells or their precursors.

Other preferred embodiments include (preferably prior to administering the stem cells): inactivating the natural killer cells of the recipient mammal, e.g., by introducing into the recipient mammal an antibody capable of binding to natural killer cells of the recipient; inactivating the T cells of the recipient mammal, e.g., by introducing into the recipient an antibody capable of binding to T cells of the recipient.

Other preferred embodiments include: the step of creating hematopoietic space, e.g., by one or more of, irradiating the recipient with low dose, e.g., between about 100 and 400 rads, whole body irradiation, administering a myleosuppressive drug to the recipient, or administering anti-class I antibodies to the recipient, to deplete or partially deplete the bone marrow of the recipient; the method includes the a step which creates hematopoietic space and the step is performed prior to introducing the transgenic cells into the recipient.

Other preferred embodiments include inactivating thymic T cells by one or more of: (preferably prior to hematopoietic stem cell transplantation) irradiating the recipient mammal with, e.g., about 700 rads of thymic irradiation; administering one, or preferably two or more, doses of an anti-T cell antibody; or administering to the recipient a short course of an immunosuppressant as described in USSN 08/220,371, filed March 29, 1994.

Other preferred embodiments include: the step of depleting or otherwise inactivating natural antibodies in the blood of the recipient mammal, e.g., by hemoperfusing an organ, e.g., a liver or a kidney, obtained from a pig or administering a drug, e.g., deoxyspergualin (DSG) which inactivates or depletes natural antibodies; the method includes a step which depletes or otherwise inactivates natural antibodies in the blood of the recipient and the step is performed prior to hematopoietic stem cell transplantation.

In preferred embodiments, the method includes the step of introducing into the recipient a graft obtained from the donor which is obtained from a different organ than the hematopoietic stem cells, e.g., a liver or a kidney.

Genetically engineered swine cells of the invention can be made by methods known to those skilled in the art, e.g., by retroviral transduction of swine cells. Methods for producing transgenic swine of the invention use standard transgenic technology. These methods include, e.g., the infection of the zygote or organism by viruses including retroviruses; the infection of a tissue with viruses and then reintroducing the tissue into an animal; and the introduction of a recombinant nucleic acid molecule into an embryonic stem cell of a mammal followed by appropriate manipulation of the embryonic stem cell

to produce a transgenic animal. In particular, the invention features a transgenic swine, whose germ cells and somatic cells contain a transgene including a DNA sequence encoding a hematopoietic peptide and a tissue-specific promoter operably linked to the DNA sequence, wherein the tissue-specific promoter effects expression of the hematopoietic peptide in bone marrow cells of the swine, the transgene being introduced into embryonal cells of the animal, or an ancestor of the animal.

Yet another aspect of the invention features a method for identifying or testing an agent, e.g., a therapeutic agent, e.g., an agent useful in treating a hematopoietic disorder, by evaluating the agent's effect on transgenic swine cells of the invention. In an illustrative embodiment, an agent is administered to a transgenic swine, and the state of a hematopoietic tissue, an aspect of metabolism, or an aspect of gene expression, evaluated and compared with that of a control standard, e.g., that of a control transgenic animal. The present method may be employed, for example, to determine the *in vivo* efficacy of agonists or antagonists of human hematopoietic growth factors. Analogous experiments can be performed with cultured cells.

It has been demonstrated that xenogeneic donor hematopoietic stem cells, when engrafted in xenogeneic recipients, can induce a state of donor-specific tolerance to transplanted donor organs. Even low levels of hematopoietic chimerism can be sufficient to induce this tolerant state. However, the loss of chimerism (which often occurs) is associated with a concomitant loss of tolerance. The animals, cells, transgenes, and methods of the invention can be used to promote the formation and maintenance of chimerism. The transgenes, transgenic cells, transgenic animals, and methods of the invention are also useful in drug testing protocols, e.g., in protocols for identifying agents which interact with human receptor or adhesion molecules, e.g., for identifying agents which act as agonists or antagonists of human growth factors or adhesion molecules. The transgenes, transgenic cells, transgenic animals and methods of the invention are also useful for determining the species specificity of an interaction between a human receptor or adhesion molecule and a swine ligand.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description of the Invention

The drawings will first be briefly described.

Drawings

Fig. 1 is a diagram of the GS4.5 retroviral construct.

Fig. 2 is a diagram of the GS4.5 proviral genome and the expected transcripts.

Figs. 3a and 3b are representations of flow cytometry profile of transduced cells.

Fig. 4 is a diagram of the transduction assay.

I. Repopulation of Xenogeneic Bone Marrow

Embodiments of the invention relate to genetically engineered swine cells, e.g., to genetically engineered hematopoietic stem cells, which express recombinant peptides, e.g., human peptides. The peptides enhance any of: the survival, engraftment, proliferation, or function of swine cells implanted in a xenogeneic host.

In an exemplary embodiment the survival, engraftment, proliferation, or function of stem cells, or the development of the stem cells, into differentiated cell types of the cells, in the presence of a human hematopoietic environment, such as found in human hematopoietic tissues, e.g., bone marrow, is promoted. The engineered cells can express, e.g., a human growth factor receptor, e.g., a human growth factor involved in the control of hematopoiesis. Examples of human growth factor receptors include the receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, and uteroferrin. In other exemplary embodiments, the engineered cells express a human adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells. Examples of such molecules include VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, and CD34.

Competitive repopulation studies of discordant bone marrow transfer have shown that xenogeneic hematopoietic cells can be at a competitive disadvantage for reconstitution of a xenogeneic host, especially when transplanted as a part of a non-myleoablative regimen. That is, when stem cells of the host are available, the host cells will generally have a competitive advantage in reconstitution of the host. This competitive advantage can be a major factor preventing reconstitution by engrafted xenogeneic hematopoietic stem cells. Several factors may be responsible for the competitive advantage enjoyed by autologous bone marrow over engrafted xenogeneic stem cells. The advantage appears to derive in part, from an inability of the xenogeneic cells to adequately engage growth factors and extracellular matrix (ECM) components of the host.

Both extracellular matrix components and growth factors play important roles in the regulation of hematopoiesis and the maintenance of both stem and differentiated hematopoietic cells, and therefore are of consequence to the survival of xenogeneic cells. Stem cell maintenance, for example, require growth factor binding as well as close range interactions with surrounding cells for engraftment, e.g. stromal cells. However, both ECM interactions and growth factor binding by hematopoietic cells can be, at least partially, species dependent, such that in xenogeneic settings, the grafted marrow is at a competitive disadvantage relative to autologous marrow in at least one of mitogenic stimulation or engraftment. For example, the species specificity of adhesion molecule interactions (such as mediated by VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, or CD34), are thought to be

important in hematopoiesis. The failure of such adhesion molecules to interact with ligands from the host species may be a major impediment to homing and reconstitution by xenogeneic stem cells. Likewise, a number of growth factors, e.g. hematopoietic growth factors, are known to show varying degrees of species of specificity in their receptor interactions. For instance, G-CSF displays significant, though not absolute, species specificity. IL-3, for example, has a very marked species specificity.

In an illustrative embodiment, described in further detail below, the human c-kit receptor (hereinafter "c-kit"), the ligand for SCF, is engineered into swine stem cells. Because the human c-kit bearing stem cells can interact effectively with human SCF bound to stromal cells, and because human SCF can act as growth factor for this recombinant swine stem cells, some of the competitive advantage enjoyed by human marrow would be lost to the engrafted swine stem cells Thus, the use of c-kit humanized swine cells can facilitate improved reconstitution of a human subject by swine stem cells. A similar approach can be used for human hematopoietic growth factor receptors, such as IL-3 receptors and GM-CSF receptors, as well as for human adhesion molecules that are involved in hematopoiesis and for which swine cells do interact effectively with the human ligand. Thus, in one aspect of the invention, the subject "humanized" swine cells can be used in xenografting protocols. By virtue of an improved ability to respond to human factors which regulate hematopoietic cells (relative to the wild-type swine cell), the humanized cells can be used to improve engraftment and/or survival of swine hematopoietic stem cells, and thereby promote tolerance, in humans subjects. Preferred embodiments of the present invention include a method of providing a growth selective advantage to a transgenic cell, relative to the corresponding wild-type cell, when used as a source of xenogeneic graft tissue, by providing a swine cell which expresses a human hematopoietic peptide, e.g., by producing a transgenic mammal having at least 1 cell containing and expressing a recombinant nucleic acid molecule of the present invention. The recombinant nucleic acid molecule containing transgenic mammal is maintained for a time period sufficient for the hematopoietic modulator gene present in the recombinant nucleic acid molecule to be expressed in the cell and thereby provide a selective advantage to the transgenic cell, relative to the corresponding wild-type cell, when transplanted into another species. In another aspect, the recombinant swine cells, particularly transgenic swine bearing such cells, are useful for assaying the efficacy of recombinant human hematopoietic factors.

As used herein, a graft-supporting protein refers to a protein which has one or more of the following properties: when expressed in a swine cell, it prolongs or otherwise promotes the acceptance of that cell in a xenogeneic donor; when expressed in a swine cell, it prolongs or otherwise promotes the acceptance of another swine cell in a xenogeneic donor; when expressed in a swine cell, it increases or otherwise promotes the

function of that swine cell in a xenogeneic donor; when expressed in a swine cell, it increases or otherwise promotes the function of another swine cell in a xenogeneic donor. The graft-supporting protein can be expressed either, or both, prior to, or after, implantation of the cell or tissue it affects is implanted in the xenogeneic host. The graft-supporting protein can exert its action on a swine cell or tissue either or both, prior to or after the affected cell or tissue is implanted in a xenogeneic recipient. E.g., the graft-supporting protein can be expressed in a cultured cells, and then, graft-supporting protein expressing cultured cells implanted (alone or with other swine tissue) in a donor; the graft-supporting protein can be expressed in a transgenic swine, and graft-supporting protein expressing cells from the transgenic swine implanted (alone or with other swine tissue) in a donor. (An affected cell or tissue is a cell or tissue upon which the graft-supporting protein has a direct or indirect (through its action on another cell) effect; the affected cell or tissue can be the cell or tissue which expresses the graft-supporting protein, or a cell which does not express the graft-supporting protein, e.g., a cell or tissue implanted together with a graft-supporting protein expressing cell.) Examples of graft-supporting proteins include: recipient HLA molecules; recipient growth factor receptors; recipient adhesion molecules; and recipient or donor proteins related to the function of the graft. Graft-supporting proteins include hematopoietic proteins.

As used herein, a graft-antagonistic protein is a protein the expression of which, by the graft tissue or by the recipient, promotes an immune response directed against donor tissue or against the recipient, or is otherwise antagonistic to the function of the stem cells or graft or is otherwise antagonistic to the acceptance of the donor stem cells or the graft by the recipient. Examples of graft antagonistic proteins are donor cell surface receptors for host proteins which mediate an immune response against the donor cell, e.g., the donor cell B7, CD27, or LFA-3 receptors, or a donor receptor for a host cytokine.

As used herein, the term "transgene" means a nucleic acid sequence (encoding, e.g., one or more hematopoietic peptides), which is partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic pig or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can include one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal expression of the selected nucleic acid, all operably linked to the selected nucleic acid, and may include an enhancer sequence.

As used herein, the term "transgenic cell" refers to a cell containing a transgene.

As used herein, a "transgenic animal" is any animal in which one or more, and preferably essentially all, of the cells of the animal includes a transgene. The transgene is

introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or *in vitro* fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the transgenic animals described herein, the transgene causes cells to express a recombinant peptide, (e.g., a recombinant hematopoietic peptide, e.g., a human growth factor or cytokine receptor involved in regulation of hematopoiesis, such as receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, or uteroferrin, or a human adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells, such as VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, or CD34), in cells that do not express such hematopoietic peptides in a wild-type, non-transgenic animal. Transgenic swine which include one or more transgenes encoding one or more human hematopoietic peptides are within the scope of this invention. For example, a double or triple transgenic animal, which includes two or three transgenes can be produced.

As used herein, the term "germ cell line transgenic animal" refers to a transgenic animal in which the transgene genetic information exists in the germ line, thereby conferring the ability to transfer the information to offspring. If such offspring in fact possess some or all of that information then they, too, are transgenic animals.

The term "hematopoietic gene" is used herein to mean any gene whose gene product, preferably a human gene product, which when expressed by a swine hematopoietic cells, is capable of enhancing the ability of a swine hematopoietic stem cell to competitively reconstitute a primate, e.g., a human host. For example, the human-gene product can enhance the proliferative ability of recombinant swine cells in human subjects; increase the ability of swine cells to bind to extracellular matrix components; or enhance the functional activity of swine marrow cells. In preferred embodiments, the human hematopoietic gene encodes: a cell surface protein; a human hematopoietic growth factor receptor, such as receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo; or uteroferrin, or a human adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells, such as VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, or CD34. The gene products of human hematopoietic genes are referred to herein as "human hematopoietic proteins or peptides (the terms protein, peptide, and polypeptide are in this sense used interchangeably)", and include human hematopoietic growth factor receptors and human hematopoietic adhesion molecules. The term "growth factor", or "hematopoietic growth factor", is used to describe biologically active molecules which can, for example,

stimulate proliferation of the recombinant swine cell, enhance binding to ECM components and/or increase the functional activity of the cell. The term "cytokine" is used interchangeably with growth factor. Examples of hematopoietic growth factors include G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, and uteroferrin. "Growth factor receptors" are protein(s) expressed by cells, typically on the extracellular surface, which facilitate binding of growth factors by the cell and which alone, or in conjunction with other cellular proteins, induce a biological response in the cell to the binding of the growth factor. Hematopoietic genes include those which encode a hybrid protein, e.g., a peptide which encodes both human and swine components, e.g., a hybrid receptor with a human extracellular domain and a swine intracellular or transmembrane domain. A hematopoietic protein is a protein encoded by a hematopoietic gene.

As used herein, the term "operably linked" means that selected DNA, e.g., encoding a hematopoietic peptide, is in proximity with a transcriptional regulatory sequence, e.g., tissue-specific promoter, to allow the regulatory sequence to regulate expression of the selected DNA.

The term "genetically programmed" as used herein means to permanently alter the DNA, RNA, or protein content of a cell. Typically, this genetic programming is accomplished by introducing into a swine cell a recombinant nucleic acid molecule which encodes a hematopoietic peptide.

As used herein, the term "recombinant swine cells" refers to cells derived from swine, preferably miniature swine, which have been used as recipients for a recombinant vector or other transfer nucleic acid, and include the progeny of the original cell which has been transfected or transformed. In a preferred embodiment, the recombinant swine cell is derived from a swine hematopoietic stem cell. e.g., a swine bone marrow hematopoietic cell, and has been genetically programmed to express a recombinant human peptide. Recombinant swine cells include cells in which transgenes or other nucleic acid vectors have been incorporated into the host cell's genome, as well as cells harboring expression vectors which remain autonomous from the host cell's genome.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, e.g. the transformed swine cell expresses human cell surface peptides.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/expression of nucleic

acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors".

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of the recombinant hematopoietic gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which naturally controls the expression of the recombinant gene in humans, or which naturally controls expression of the corresponding gene in swine cells. In even more preferred embodiments, the transcription regulatory sequence causes hematopoietic-specific expression of the recombinant protein. The above embodiments not withstanding, it will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences different from those sequences naturally controlling transcription of the recombinant protein. Transcription of the recombinant gene, for example, can be under the control of a synthetic promoter sequence. Preferably, the promoter sequence controlling transcription of the recombinant gene is active (i.e., can promote gene expression) in bone marrow cells, especially hematopoietic cells. The promoter that controls transcription of the recombinant gene may be of viral origin; examples are promoters sometimes derived from bovine herpes virus (BHV), Moloney murine leukemia virus (MLV), SV40, Swine vesicular disease virus (SVDV), and cytomegalovirus (CMV).

As used herein, the term "tissue-specific promoter" means a DNA sequence that serves as a promoter, i.e., regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in specific cells, e.g., hematopoietic cells. The tissue specific promoter directs expression predominantly, if not exclusively in hematopoietic cells. Particularly useful promoter sequences for directing expression of human hematopoietic genes include: promoter sequences naturally associated with the recombinant human gene; promoter sequences naturally associated with the homologous pig gene (i.e. corresponding to the recombinant human gene); promoters which are active primarily in hematopoietic cells, e.g. in lymphoid cells, in erythroid cells, or in myeloid cells; the immunoglobulin promoter described by Brinster et al. (1983) *Nature* 306:332-336 and Storb et al. (1984) *Nature* 310:238-231; the immunoglobulin promoter described by Ruscon et al. (1985) *Nature* 314:330-334 and Grosscheld et al. (1984) *Cell* 38:647-658; the globin promoter described by Townes et al. (1985) *Mol. Cell. Biol.* 5:1977-1983, and Magram et al. (1989) *Mol. Cell. Biol* 9:4581-4584. Other hematopoietic promoters are described herein or will be apparent to those skilled in the art, and may include regulatory sequences derived from such lymphoid genes as CD1, CD2, CD3-γ, CD3-δ, CD3-ε, CD3-ζ, CD3-η, CD4, CD5,

CD7, CD8, CD19, CD20, CD38, CD40, CD45, CD72, CD76, $p56^{lck}$, IL-2Rβ chain, J11d (heat stable antigen), fyn, NK1, NK2, Fc RIγ chain, IL-2R β-chain, αTCAR, βTCAR, γ TCAR, δTCAR, $Fc_\gamma RIII$, RAG-1, RAG-2, Ig-β (B29), or IgM-α (MB-1) and genes associated with immunoglobulin isotypes Igμ, Igδ, Igγ, Igα, Igε; Igk and Igλ. Moreover, such promoters also may include additional DNA sequences that are necessary for expression, such as introns and enhancer sequences. The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well. Other regulatory elements e.g., locus control regions, e.g., DNase I hypersensitive sites, can be included.

By "cell specific expression", it is intended that the transcriptional regulatory elements direct expression of the recombinant protein in particular cell types, e.g., bone marrow cells. The term "hematopoietic specific expression" therefore refers to expression of a recombinant protein which is substantially restricted to hematopoietic cells.

"Graft", as used herein, refers to a body part, organ, tissue, or cells. Grafts may consist of organs such as liver, kidney, heart or lung; body parts such as bone or skeletal matrix; tissue such as skin, intestines, endocrine glands; or progenitor stem cells of various types.

The term "tissue" as used herein, means any biological material that is capable of being transplanted and includes organs (especially the internal vital organs such as the heart, lung, liver, kidney, pancreas and thyroid), cornea, skin, blood vessels and other connective tissue, cells including blood and hematopoietic cells, Islets of Langerhans, brain cells and cells from endocrine and other organs and bodily fluids, all of which may be candidate for transplantation.

"A discordant species combination", as used herein, refers to two species in which hyperacute rejection occurs when a graft is grafted from one to the other. In the subject invention, the donor is of porcine origin and the recipient is human.

"Hematopoietic stem cell", as used herein, refers to a cell, e.g., a bone marrow cell, a fetal or neonatal liver or spleen cell, or a cord blood cell which is capable of developing into a mature myeloid and/or lymphoid cell.

"Progenitor cell", as used herein, refers to a cell which gives rise to an differentiated progeny. In contrast to a stem cell, a progenitor cell is not always self renewing and is relatively restricted in developmental potential.

As used herein, the term "hematopoietic cells" embraces differentiated blood cells, including: cells derived from a myeloid lineage, including megakaryocytes, monocytes, granulocytes, and eosinophils; cells derived from an erythroid lineage, such as red blood cells; and cells of a lymphoid lineage such as B lymphocytes and T lymphocytes. As is generally understood, each of the above lineages mature from "pluripotent hematopoietic stem cells" (also referred to herein as "hematopoietic stem cells" or "colony-forming stem

cells"), which undergo a series of differentiation steps leading to increasingly lineage-restricted progenitor cells. Thus, the term hematopoietic cell also encompasses the various hematopoietic precursor cells from which these differentiated cells develop, such as BFU-E (burst-forming units-erythroid), CFU-E (colony forming unit-erythroid), CFU-Meg (colony forming unit-megakaryocyte), CFU-GM (colony forming unit-granulocyte-monocyte), CFU-Eo (colony forming unit-eosinophil), and CFU-GEMM (colony forming unit-granulocyte-erythrocyte-megakaryocyte-monocyte).

"Stromal tissue", as used herein, refers to the supporting tissue or matrix of an organ, as distinguished from its functional elements or parenchyma.

"Tolerance", as used herein, refers to the inhibition of a graft recipient's immune response which would otherwise occur, e.g., in response to the introduction of a nonself MHC antigen into the recipient. Tolerance can involve humoral, cellular, or both humoral and cellular responses. Tolerance, as used herein, refers not only to complete immunologic tolerance to an antigen, but to partial immunologic tolerance, i.e., a degree of tolerance to an antigen which is greater than what would be seen if a method of the invention were not employed.

"MHC antigen", as used herein, refers to a protein product of one or more MHC genes; the term includes fragments or analogs of products of MHC genes which can evoke an immune response in a recipient organism. Examples of MHC antigens include the products (and fragments or analogs thereof) of the human MHC genes, i.e., the HLA genes. MHC antigens in swine, e.g., miniature swine, include the products (and fragments and analogs thereof) of the SLA genes, e.g., the DRB gene.

"Miniature swine", as used herein, refers to wholly or partially inbred animal.

As described herein, the transgenic donor tissue may come from a cell culture or from a transgenic swine. The transgenic swine should express (or be capable of expressing) the recombinant human gene in at least the tissue to be transplanted.

The practice of the present invention will employ, unless otherwise indicated, techniques which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press:1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No. 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu

et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. All publications mentioned herein are incorporated by reference. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

II. Recombinant Human Genes.

Introduction of a human hematopoietic gene into a swine cell of the present invention requires that the recombinant human gene be transfected into the cell, or a precursor of the cell. As will be understood, the mode of introduction and the desired integration phenotype of the resulting swine cell (i.e. whether the vector is integrated into the cell's genome or remains autonomous) can influence the choice of expression vector used to generate the subject recombinant swine cells. In general, expression vectors containing the human hematopoietic gene can be constructed by operably linking an appropriate transcriptional regulatory sequence, e.g. a tissue-specific promoter, with a nucleic acid, e.g. cDNA or genomic DNA, encoding the hematopoietic protein. Moreover, a tissue-specific promoter can be linked to more than one cDNA, each encoding a different human hematopoietic protein, or a human hematopoietic protein and some other foreign protein, e.g., another cell surface antigen. Depending on the specific promoter used, it may be desirable to modify the promoter-cDNA construct to include an intron splice site and/or a polyadenylation signal.

In addition to the 5' and 3' expression regulation sequences and the recombinant DNA (either genomic or derived from cDNA) the transgenes of the invention can also include a "recombinant intervening sequence" which interrupts the transcribed but untranslated 5' region of the transgene. Such intervening sequences (IVS) are known in the art. Such sequences as used herein are "homologous recombinant intervening sequences" in that the 5' and 3' RNA splice signals in the IVS are those normally found in an IVS from an endogenous or heterologous gene. Recombinant intervening sequences may, however, also comprise a "hybrid intervening sequences". Such hybrid intervening sequences comprise a 5' RNA splice signal and 3' RNA splice signal from intervening sequences from different sources. In some aspects of the invention, such hybrid IVS comprise at least one "permissive RNA splice sequence". As used herein, a permissive RNA splice signal is an RNA splice signal sequence, preferably a 3' RNA splice signal, from an intron contained within a repertoire of germ line DNA segments which undergo rearrangement during cell differentiation. Examples of such gene repertoires include the

immunoglobulin super gene family, including the immunoglobulins and T-cell antigen receptors as well as the repertoire of the major histocompatibility complex (MHC) genes and others. Particularly preferred permissive splice sequences are those obtained from the immunoglobulin repertoire, preferably of the IgG class, and more preferably those 3' splice signal sequences associated with the J-C segment rearrangement of the Ig heavy and light chain, most preferably the heavy chain. Hybrid intervening sequences containing permissive RNA splice signals are preferably used when the recombinant DNA corresponds to a cDNA sequence.

Based on the foregoing, it is apparent that preferred transgenes can include relatively large amounts of 5' and 3' expression regulation sequences. Further, the recombinant DNA is preferably derived from genomic clones which may be tens to hundreds of kilobases in length. Based on the present technology for cloning and manipulating DNA, the construction and microinjection of transgenes is practically limited to linearized DNA having a length not greater than about 100kb. However, the transgenes of the invention, especially those having a length greater than about 50kb, may be readily generated by introducing two or more overlapping fragments of the desired transgene into an embryonal target cell. When introduced, the overlapping fragments undergo homologous recombination which results in integration of the fully reconstituted transgene in the genome of the target cell. In general, it is preferred that such overlapping transgene fragments have 100% homology in those regions which overlap. However, lower sequence homology may be tolerated provided efficient homologous recombination occurs. If non-homology does exist between the homologous sequence portions, it is preferred that the non-homology not be spread throughout the homologous sequence portion but rather be located in discrete areas. Although as few as 14 base pairs at 100% homology are sufficient for homologous recombination in mammalian cells (Rubnitz et al. (1984) *Mol. Cell. Biol.* 4:2253-2258), longer homologous sequence portions are preferred, e.g. 500bp, more preferably 1,000bp, next most preferably 2,000bp and most preferably greater than 2,000bp for each homologous sequence portion. It may also be desirable to use YAC's and MAC's for manipulation of recombinant nucleic acids of the invention.

In further embodiments, the recombinant hematopoietic protein can be a chimeric peptide having a portion encoded by a human hematopoietic gene, and a portion encoded by a swine hematopoietic gene. In a preferred embodiment, the chimeric protein comprises an extracellular domain of human origin, and an intracellular domain (and transmembrane domain) of swine origin. Such a chimeric protein can be useful in circumstances such as where the intracellular domain of the human molecule is less efficient than the swine counterpart at engaging intracellular signal transduction proteins of the swine cell.

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments (e.g. between the human gene and the swine gene) which can subsequently be annealed to generate a chimeric gene sequence (see, for example, Current Protocols in Molecular Biology, Eds. Ausubel et al. John Wiley & Sons: 1992).

Since the mid-1980's, a number of hematopoietic growth factor receptors have been cloned from human sources and are readily accessible by standard molecular biological techniques for use in the present invention (see, for review, Foxwell et al. (1992) *Clin Exp Immunol* 90: 161-169). In many instances, the transcriptional regulatory sequences naturally associated with the cloned genes have likewise been identified and well characterized. Moreover, in light of the present disclosure, it will be apparent to one skilled in the art that other suitable genes can be obtained from human sources, and swine transcriptional regulatory sequences obtained and used with the human gene.

In one aspect of the invention, the recombinant swine cells are derived to express the human c-kit gene, or another member of the closely related tyrosine kinase receptor family to which it belongs, such as the colony-stimulating factor I receptor (c-fms), a platelet-derived growth factor receptor (PDGF-Ra and PDGF-RB), or a fetal liver kinase (FLK-1 or FLK-2) (see, for review, Andre et al. (1992) *Oncogene* 7:685-691). cDNA clones, and in some instances genomic clones, of each of human c-kit (Ogawa et al. (1994) *Exp Hematol* 22:45-51; Vardenbark et al. (1992) *Oncogene* 7:1259-1266; Yarden et al. (1987) *EMBO J* 6:3341-3351; Blume-Jensen et al. (1991) *EMBO J* 10:4121-4128), human c-fms (Bourette et al. (1993) *Blood* 81: 2511-2520; Dibbs et al. (1990) *Growth Factors* 2:301-311; Sherr (1988) Leukemia 2:1325-1425), human PDGF-Rα and human PDGF-Rb (Matsui et al. (1989) *DNAS* 86:8314-8318), and human FLK-1 and human FLK-2 (U.S. Patent No. 5,270,458; PCT Publication No. WO 93/10136; PCT Publication No. WO 93/00349; U.S. Patent No. 5,283,354) have been isolated, and as described below, can be used to generate the expression vectors, including transgenes, required to derive the subject swine cells. Moreover, the promoter sequences, and other transcriptional regulatory sequences, have been characterized for each receptor (Yasuda et al. (1993) *Biochem Biophys Res Commun* 191:893-901; Yamamoto et al. (1993) *Jpn J Cancer Res* 84:1136-1144), and can be utilized in the recombinant hematopoietic gene construct. Alternatively, the regulatory sequences flanking the corresponding swine gene

(genomic) can be cloned by standard techniques and employed to regulate expression of the human gene in the recombinant swine cell.

In another aspect of the invention, the recombinant swine cells are engineered to express the human Granulocyte-Macrophage Colony-Stimulating factor (GM-CSF) receptor. The human GM-CSF receptor has been cloned (Sasaki et al. (1993) *J Biol Chem* 268:13697-13702; Sakamaki et al (1992) *EMBO J* 11:3541-3549; Hayashida et al. (1992) *Nippon Rinsho* 50:1867-1871; Metcalfe et al. (1990) *PNAS* 87:4670-4674; Gearing et al (1989) *EMBO J* 8:3667-3676; Lock et al. (1994) *PNAS* 91:252-256 and Kitamura et al. (1991) *PNAS* 88:5082-5086), and found to consist of two polypeptide chains, the "alpha chain" and the "beta chain", the latter of which is utilized by other growth factor receptor complexes, such as the IL-3 receptor and the IL-5 receptor. Each of the two chains can be expressed from the same expression vector, or from two separate expression vectors within the same cell. A transgenic swine which express a first transgene can be crossed with a transgenic swine which expresses a second transgene to provide a transgenic animal which expresses both. (Modifications of this technique can be used to add third and subsequent genes as well.) Thus, a transgenic swine which expresses only one transgene or the other of the alpha or beta chains, can be cross-bred with the appropriate transgenic mate to yield offspring which are chimeric for both chains. Alternatively, only the alpha chain need be expressed as it may form active receptor complexes with the swine beta chain. The alpha chain provides most of the binding specificity to the receptor complex and is therefor more likely to influence species specific binding of GM-CSF than is the beta chain.

In similar fashion, human IL-3 receptors can be constituted in swine hematopoietic cells utilizing the cloned genes for each of the alpha and beta chain subunits (Sakamaki et al. (1992) *EMBO J* 11:3541-3549; and Kitamura et al. (1991) *611* 661165-1174) to derive appropriate expression vectors.

Thus, a nucleotide sequence derived from the cloning of the human hematopoietic gene, encoding all or a selected portion of the human protein, can be used to produce a recombinant form of the human protein in swine cells. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into swine cells can be carried out by standard procedures.

The recombinant nucleic acid constructs described above may be inserted into any suitable plasmid, bacteriophage, or viral vector for amplification, and may thereby be propagated using methods known in the art, such as those described in *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press:1989). In the preferred embodiments, expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells are used. Eukaryotic cell expression vectors are well known in the art and are available from

several commercial sources. The preferred swine expression vectors contain both prokaryotic sequences (to facilitate the propagation of the vector in bacteria), and one or more eukaryotic transcription units that are functional in swine cells. Typically, such vectors provide convenient restriction sites for insertion of the desired recombinant DNA molecule. The pcDNAI, pSV2, pSVK, pMSG, pSVL, pPVV-1/PML2d and pTDT1 (ATCC, No. 31255) derived vectors are examples of mammalian expression vectors suitable for transfection of swine cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for expression of proteins in swine cells. The various methods employed in the preparation of the plasmids and transformation of host cells are well known in the art. For other suitable expression systems for useful in the present invention, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press:1989).

Under those circumstances wherein the recombinant nucleic acid molecule is introduced into swine oocytes as a transgene to be incorporated into the host genome, the construct can be linearized and excess vector sequences will preferably be removed, for example, by cutting the recombinant nucleic acid molecule with one or more restriction endonucleases to produce a linear nucleic acid molecule containing as a minimum, the desired transcriptional regulatory sequences and a human hematopoietic gene. Preferably the nucleic acid molecule (e.g. the transgene) is from about 5,000 base pairs to about 100,000 base pairs in length.

The suitability of particular human hematopoietic proteins, e.g. whether the human protein is more appropriate than its swine counterpart, can be determined using one of the assays described below, and its cDNA can then be determined by standard techniques. For example, according to the invention, one would link a promoter with cDNA encoding human c-kit to create a vector for expression of human c-kit in swine cells. The expression of this promoter-hematopoietic protein transgene can be verified, for example, by direct detection of human c-kit expression in appropriate tissue culture cells. Additionally, the ability of the human gene to potentially provide improved *in vivo* survival of the transgenic swine cells can be predicted *in vitro* by comparing the relative biologic activities of the human versus the endogenous protein in swine cells. Improved survival should be correlated with the ability of the recombinant cell to grow in semisolid media in response to a human growth factor.

Some transgenic pigs may carry multiple copies of the transgene, with the transgene copies incorporated at different sites in the genome. The site of transgene

incorporation into the genome can strongly influence transgene expression; therefore, one may correlate transgene expression with discrete transgene restriction fragment length polymorphism patterns. In addition, as discussed above, two transgenic swine, each expressing a different hematopoietic protein, or one hematopoietic protein and some other foreign antigen. e.g. a human MHC peptide, on the same, or different, tissue cells, can be mated to produce an animal that expresses both transgene products. The same effect can be achieved by introducing two separate transgenes into the same embryonal cell.

Exemplary *in vitro* assays useful for determining whether a particular human hematopoietic protein is suitable for use in methods of the invention include: (1) assays that measure binding of the ligand, e.g. growth factor of ECM component, to the extracellular domain of the human hematopoietic protein on the cell surface of the recombinant swine cell; and (2) assays that test for activation of the signal transduction pathways that are activated by the interaction of the human cell surface protein and an agonistic ligand. The first class of assays is useful to, for example, identify potential human hematopoietic proteins which may improve viability of xenografted swine marrow by comparing the binding constant of the human ligand (e.g. SCF, IL-3, GM-CSF) for the recombinant swine cell versus the naturally occurring swine cell. The second class of assays is preferred to determine which of the human hematopoietic proteins are suitable for use in the present invention based upon their ability to function in the swine cell.

III. Genetically Engineered Swine Cells

Transgenic swine cells of the invention can be produced by any methods known to those in the art. Transgenes can be introduced into cells, e.g., stem cells, e.g., cultured stem cells, by any methods which allows expression of these genes at a level and for a period sufficient to promote engraftment or maintenance of the cells. These methods include e.g., transfection, electroporation, particle gun bombardment, and transduction by viral vectors, e.g., by retroviruses. Transgenic swine cells can also be derived from transgenic animals.

Retroviral Introduction of Transgenes

Recombinant retroviruses are a preferred delivery system. They have been developed extensively over the past few years as vehicles for gene transfer, see e.g., Eglitis et al., 1988 , *Adv. Exp. Med. Biol.* 241:19 . The most straightforward retroviral vector construct is one in which the structural genes of the virus are replaced by a single gene which is then transcribed under the control of regulatory elements contained in the viral long terminal repeat (LTR). A variety of single-gene-vector backbones have been used, including the Moloney murine leukemia virus (MoMuLV). Retroviral vectors which permit multiple insertions of different genes such as a gene for a selectable marker and a second gene of interest, under the control of an internal promoter can be derived from this type of backbone, see e.g., Gilboa, 1988, *Adv. Exp. Med. Biol.* 241:29.

The elements of the construction of vectors for the expression of a protein product are known to those skilled in the art. The most efficient expression from retroviral vectors is observed when "strong" promoters are used to control transcription, such as the SV 40 promoter or LTR promoters, reviewed in Chang et al., 1989, Int. J. Cell Cloning 7:264. These promoters are constitutive and do not generally permit tissue-specific expression. Other suitable promoters are discussed above.

The use of efficient packaging cell lines can increase both the efficiency and the spectrum of infectivity of the produced recombinant virions, see Miller, 1990, *Human Gene Therapy* 1:5. Murine retroviral vectors have been useful for transferring genes efficiently into murine embryonic, see e.g., Wagner et al., 1985, *EMBO J.* 4:663; Griedley et al., 1987 *Trends Genet.* 3:162, and hematopoietic stem cells, see e.g., Lemischka et al., 1986, *Cell* 45:917-927; Dick et al., 1986, *Trends in Genetics* 2:165-170.

A recent improvement in retroviral technology which permits attainment of much higher viral titers than were previously possible involves amplification by consecutive transfer between ecotropic and amphotropic packaging cell lines, the so-called "ping-pong" method, see e.g., Kozak et al., 1990, *J. Virol.* 64:3500-3508; Bodine et al., 1989, *Prog. Clin. Biol. Res.* 319: 589-600.

Transduction efficiencies can be enhanced by pre-selection of infected marrow prior to introduction into recipients, enriching for those bone marrow cells expressing high levels of the selectable gene, see e.g., Dick et al., 1985, *Cell* 42:71-79; Keller et al., 1985, *Nature* 318:149-154. In addition, recent techniques for increasing viral titers permit the use of virus-containing supernatants rather than direct incubation with virus-producing cell lines to attain efficient transduction, see e.g., Bodine et al., 1989, *Prog. Clin. Biol. Res.* 319:589-600. Because replication of cellular DNA is required for integration of retroviral vectors into the host genome, it may be desirable to increase the frequency at which target stem cells which are actively cycling e.g., by inducing target cells to divide by treatment *in vitro* with growth factors, see e.g., Lemischka et al., 1986, *Cell* 45:917-927, a combination of IL-3 and IL-6 apparently being the most efficacious, see e.g., Bodine et al., 1989, *Proc. Natl. Acad. Sci.* 86:8897-8901, or to expose the recipient to 5-fluorouracil, see e.g., Mori et al., 1984, *Jpn. J. Clin. Oncol.* 14 Suppl. 1:457-463, prior to marrow harvest, see e.g., Lemischka et al., 1986, *Cell* 45:917-927; Chang et al., 1989, *Int. J. Cell Cloning* 7:264-280.

The inclusion of cytokines or other growth factors in the retroviral transformations can lead to more efficient transformation of target cells.

Example 1

Production of retrovirally transformed cells and sustained expression of a swine transgene in murine bone marrow hematopoietic cells by retroviral-mediated gene transfer

The efficacy of a retroviral gene transfer approach for introducing expressible genes was shown by using double-copy retroviral vectors engineered to express a drug-resistance marker (neomycin) and a swine class II DRB cDNA. Although this example uses the swine class II DRB gene, those skilled in the art will recognize that other genes described herein, e.g., the human c kit gene, can be substituted therefor.

Infectious particles containing these vectors were produced at a titer of $>1 \times 10^6$ G418-resistant colony-forming units/ml using both ecotropic and amphotropic packaging cell lines. Flow cytometric analysis of DRA-transfected murine fibroblasts subsequently transduced with virus-containing supernatants demonstrated that the transferred sequences were sufficient to produce DR surface expression. Cocultivation of murine bone marrow with high-titer producer lines leads to the transduction of 40% of granulocyte/macrophage colony-forming units (CFU-GM) as determined by the frequency of colony formation under G418 selection. After nearly 5 weeks in long-term bone marrow culture, virus-exposed marrow still contained G418-resistant CFU-GM at a frequency of 25%. In addition, virtually all of the transduced and selected colonies contained DRB-specific transcripts. These results show that a significant proportion of very primitive myelopoietic precursor cells can be transduced with the DRB recombinant vector and that vector sequences are expressed in the differentiated progeny of these cells. These experiments are described in detail below.

Details of retroviral constructs are given in Fig. 1. Two types of retroviral constructs, GS4.4 and GS4.5, were prepared. The diagram in Fig. 1 depicts the GS4.5 retroviral construct. The arrows in Fig. 1 indicate the directions of transcription. In GS4.5, the orientation of DRB cDNA transcription is the same as viral transcription. In GS4.4 (not shown), the TK promoter and the DRB cDNA were inserted into the 3' LTR of N2A in the reverse orientation of transcription with respect to viral transcription and the simian virus 40 3' RNA processing signal was added. pBSt refers to Bluescript vector sequence (Stratagene). The thymidine kinase (TK) promoter was contained within the 215-base-pair (bp) Pvu II-Pst I fragment from the herpes simplex virus TK gene, McKnight, 1980 *Nucleic Acids Res.* 8:5949-5964. The simian virus 40 3' RNA processing signal was contained within the 142-bp *Hpa I-Sma I* fragment from the pBLCAT3 plasmid, Luckow et al., (1987) *Nucleic Acids Res.* 15:5490-5497, (see Fig. 1). Sequence analysis of the junctions of the promoter, the class II cDNA, and the vector sequences confirmed that the elements of the constructs were properly ligated.

These retroviral constructs were transfected into the amphotropic packaging cell line PA317, and transfectants were selected in G418-containing medium. A total of 24 and 36 clones, transfected, respectively, with the GS4.4 and GS4.5 recombinant plasmids, were tested by PEG precipitation of culture supernatants and slot-blot analysis of viral RNA. Of these, 8 and 12 clones were found, respectively, to be positive for DRB,

although the DRB signal was consistently weaker for the GS4.4-derived clones. Analysis of genomic and spliced transcripts from GS4.5 cells by dot-blot analysis of PEG-precipitated particles revealed heterogeneity among viral transcripts in various clones transfected by GS4.5. In one experiment, two clones contained DRB^+/Neo^+ viral RNA, two contained DRB^+/Neo^- RNA, two contained DRB^-/Neo^+ RNA, and one showed no class II or Neo signal. G418-resistance ($G418^r$) titer determination of supernatants from DRB-positive clones confirmed that the average titer produced by GS4.5-transfected clones (10^3-10^4 CFU/ml) was significantly higher than that of the GS4.4-transfected clones (10^2-10^3 CFU/ml). Further transduction experiments were, therefore, conducted with the best clone, named GS4.5 C4, which produced an initial $G418^r$ titer of 3×10^4 CFU/ml.

Plasmid preparation, cloning procedures, DNA sequencing, RNA preparations, Northern blots, and RNA slot blots were performed by standard methods, Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual* 2nd Ed. (Cold Spring Harbor Lab., Cold Spring Harbor). Final washes of blots were carried out in 0.1 x SSPE (1 x SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA) at 60°C for 30 min.

The packaging cell lines PA317, Miller et al., 1986, *Mol. Cell. Biol.* 6:2895-2902, GP+E-86, Markowitz et al., 1988, *J. Virol* 62:1120-1124, psiCRIP, Danos et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:6460-6464, and their derivatives were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM; GIBCO) with 10% (vol/vol) fetal bovine serum (CELLect Silver; Flow Laboratories) supplemented with 0.1 mM nonessential amino acids (Whittaker Bioproducts), antibiotics penicillin (5 units/ml), and streptomycin (5 μg/ml).

Improvement of the Viral Titer of the C4 Clone. Since recent data indicated that supernatants containing high retroviral titers were the best candidates for transducing bone marrow cells, Bodine et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:3738-3742, the titer of the C4 producer clone was increased by "ping-pong" amplification, Bestwick et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:5404-5408. Supernatant from nearly confluent C4 cultures was used to transduce GP+E-86 ecotropic packaging cells and G418 selection was applied. Forty-eight clones were isolated and screened by PEG precipitation for production of viral particles. Supernatants from 18 of these clones were DRB-positive by dot-blot analysis of viral RNA and had $G418^r$ titers between 0.5 and 3.5×10^4 CFU/ml). One positive clone was then amplified by the ping-pong technique with the amphotropic hygromycin-resistant packaging line psiCRIP. Supernatants from 48 hygromycin-resistant clones were examined for presence of DRB-positive viral RNA by PEG precipitation and their $G418^r$ titers were determined. All of the clones were positive by dot-blot analysis with the DRB probes and produced titers between 1×10^5 and 1×10^7 CFU/ml. Amphotropic clone GS4.5 A4, which produced the highest titer, was tested for

the presence of helper virus by the S + L-assay. No replication-competent helper virus was detected.

Amplification of virus titer was achieved by the ping-pong technique. Since there is evidence that psiCRIP packaging cells are less prone to produce helper virus than PA317 when using certain types of vectors, Miller, 1990, *Hum. Gene Therapy* 1:5-14, DRB recombinant virions were prepared using the psiCRIP/GP-E-86 producer combination. Titer values > 1 x 10^7 CFU/ml with no detectable amphotropic helper viruses were obtained, confirming that this strategy produced safe viral particles suitable for *in vivo* experiments.

Northern blot analysis of GS4.5-producing clones C4, A9, and A4, each derived from a different packaging cell line, showed a conserved hybridization pattern. RNA species corresponding to the full-length viral genome, the spliced Neo transcript, and the DRB transcription unit were observed with additional RNA species. High molecular size species observed in these experiments may constitute a read-through transcript starting from the TK promoter and ending in the other long terminal repeat (LTR). In contrast to many of the virion-producer clones obtained by transfection that presented erratic DRB transcripts, those obtained by transduction showed stable DRB hybridization patterns suggesting that no recombination events occurred during the amplification procedure.

Retroviral titers were determined as follows. Replication-defective retroviral particles were produced from packaging cell lines initially transfected with recombinant construct using the standard calcium phosphate precipitation method, Wigler et al., 1978, *Cell* 14:725-733. Retrovirus production was estimated by the drug-resistance titer (G418-resistant colony-forming units/ml, CFU/ml) as described, Bodine et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:3738-3742. Except for the psiCRIP line, G418 (GIBCO) selection was carried out in active component at 500 μg/ml for 10-12 days. Hygromycin B selection was applied to psiCRIP-derived packaging clones in medium containing active drug at 50 μg/ml for 10 days. Replication-competent helper virus titer was assayed on PG4 feline cells by the S^+L^- method, Bassen et al., 1971, *Nature* 229:564-566.

PEG precipitation of viral particles was performed as follows. Virions contained in 1 ml of culture supernatant were precipitated with 0.5 ml of 30% (wt/vol) polyethylene glycol (PEG) for 30 min. at 4°C. After centrifugation, the pellets were treated with a mixture of RNase inhibitors (vanadyl ribonuclease complex, BRL), phenol/chloroform-extracted, and ethanol-precipitated. Pellets were then resuspended in 15.7% (vol/vol) formaldehyde and serial dilutions were dotted onto nitrocellulose membrane.

Analysis of DRB Transcription in Packaging Cell Clones was performed as follows. To test for accurate transcription of the introduced DRB cDNA within the different producer clones, Northern blots containing RNAs isolated from these clones were hybridized with the DRB and Neo probes. Fig. 2 depicts the structure of the

provirus genome and the expected sizes of transcripts initiated from either the viral LTR or the TK promoters. Each of the three GS4.5-containing clones, which were derived from PA317 (clone C4), GP + E-86 (clone A9), and psiCRIP (clone A4) cells, showed DRB-positive transcripts. As reported, Hantzopoulos et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:3519-3523, the unspliced genomic RNA (band a) and the spliced Neo transcript (band b) were observed. In addition a transcript uniquely hybridizable with the DRB probe was detected that corresponds to the size predicted (1700 bases, band c) for the DRB cDNA transcription unit.

Surface Expression of the SLA-DR Antigen on Transduced Fibroblasts was detected as follows. An *in vitro* assay was developed to examine surface expression of the SLA-DR antigen on murine fibroblasts. Flow cytometry (FCM) profiles shown in Fig. 3 demonstrate that $G418^r$ titers of 3×10^4 (clone C4) were sufficient to promote expression of the DR antigen on the cell surface of transduced DRA transfectants. In Fig. 3 solid lines indicate DR cell surface expression (anti-DR antibody binding) (22% and 75% of the bulk population of cells 3 days after transduction with GS4.5 C4, (B) and GS4.5 A4 (C), respectively); dashed lines indicate anti-mouse class I antibody binding (positive control); dotted lines indicate anti-pig CD8 antibody binding (negative control). Twenty-two percent of the bulk population of transduced cells were DR-positive and subclones maintained class II expression for more than 5 months. The increase in titer (clone A4) correlated with an increase in the number of cells transduced (75% of the transduced population was DR-positive) and with the brightness of the DR signal.

The class II transduction assay was performed as diagrammed in Fig. 4. NIH 3T3 cells were transfected with the SLA-DRA^d cDNA inserted in a plasmid expression vector, Okayama et al., 1982, *Mol. Cell. Biol.* 2:161-170. Approximately 3×10^4 cells of a stable DRA transfectant (clone 11/12.2F) that expressed a high level of DRA mRNA were then transduced overnight with 1 ml of DRB-containing retroviral supernatant. Cells were subsequently cultivated in fresh DMEM supplemented with 10% fetal bovine serum and antibiotics for 2 additional days and examined for cell surface expression of the DR antigen by FCM analysis.

The class II transduction assay described here provides a fast and simple method to test both the expression and functional titer of retroviral constructs. By using cells transfected with DRA, the need for lengthy double selection after transduction by two separated vectors, Yang et al., 1987, *Mol. Cell Biol.* 1:3923-3928; Korman et al., 1987, *Proc. Natl. Acad. Sci. USA* 84:2150-2154, is obviated. Cell-surface expression of DR heterodimers was demonstrated by FCM analysis 3 days after transduction, providing direct evidence that the transferred sequences were sufficient to produce significant level of DR β chain. More importantly, this test allows determination of "functional" titers

based on the expression of the gene of interest rather than on that of the independently regulated drug-resistance marker.

The SLA-DRB probe was an EcoRI cDNA fragment containing the complete coding sequence of the DR β chain, Gustafsson et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:9798-9802. The neomycin phosphotransferase gene (Neo) probe was the Bcl I-Xho I fragment of the N2A retroviral plasmid, Hantzopoulos et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:3519-3523.

Expression of Porcine DRB cDNA Transduced into Murine Bone Marrow Progenitor Cells

The efficiency with which myeloid clonogenic precursors were transduced was determined by assaying for CFU-GM with and without a selecting amount of G418 after exposure of bone marrow cells to GS4.5-derived virions. Comparison of the number of colonies that formed in the presence and absence of the drug, for two experiments, indicated that ≈40% of the initial population of myeloid progenitor cells were transduced. The frequency of $G418^r$ CFU-GM was again determined after a sample of the transduced marrow was expanded under long-term culture conditions for 33 days. Twenty-five percent of the progenitors present after 33 days in culture still gave rise to colonies under G418 selection. Colonies of cells arisen from CFU-GM were examined for the presence of DRB-specific transcripts by converting RNA into cDNA and then performing PCR amplification as described herein and in Shafer et al., 1991 *Proc. Natl. Acad. Sci. USA* 88:9670. A 360-bp DRB-specific product was detected in five of six G418-selected colonies from freshly transduced marrow, whereas all six colonies similarly derived from transduced progenitors present after 33 days in culture were positive. An additional band of 100 bp observed in some of the samples probably reflects the stoichastic nature of nonspecific priming events. DRB-specific transcripts were also detected in the bulk population of drug-resistant colonies and in producer cells but were not detected in controls such as a bulk population of untransduced colonies, fibroblasts used to provide carrier RNA, and a bulk population of transduced colonies processed as above but without reverse transcriptase. These latter data demonstrate that the PCR signal was dependent on the synthesis of cDNA, excluding the possibility that provirus, rather than viral message, was responsible for the amplified fragment .

Recent improvements including modifications of the virus design, increase of viral titers, use of growth factors to stimulate precursor cells, and selection of stem cells prior to transduction have been shown to improve long-term expression of transduced genes in the hematopoietic compartment, Bodine et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:3738-3742; Bodine et al., 1989, *Proc. Natl. Acad. Sci.* USA 86:8897-8901; Wilson et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:439-443; Kang et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:9803-9807; Bender et al., 1989, *Mol. Cell. Biol.* 9:1426-1434. The

experiments herein show the applicability of the retroviral gene-transfer technique in achieving expression of major histocompatibility complex class II genes transferred into hematopoietic cells. To determine the efficiency with which developmentally primitive hematopoietic cells were transduced, the frequency of $G418^r$ CFU-GM was assessed after expanding infected marrow cells kept for 33 days in long-term cultures. Expression of the exogenous DRB cDNA was also monitored in cells derived from transduced CFU-GM present either immediately after infection or after an extended culture period. Virtually all of the colonies individually tested were positive for DRB-specific transcript, suggesting that the DRB recombinant vector is suitable for expression in murine hematopoietic cells.

Bone marrow cells were obtained from the femora of 6- to 12-week-old female C57BL/10 mice and were prepared as described, Ildstad et al., 1984, *Nature* 307:168-170. Methylcellulose colony assays for granulocyte/macrophage colony-forming units (CFU-GM), Eaves et al., 1978, *Blood* 52:1196-1210, were performed as described using 5% (vol/vol) murine interleukin 3 culture supplement (Collaborative Research). Long-term Dexter-type bone marrow cultures were initiated in 60-mm culture dishes with 2×10^7 nucleated cells, Eaves et al., 1987, *CRC Crit. Rev. Oncol. Hematol.* 7:125-138.

Bone marrow cells were transduced essentially as described, Bodine et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:8897-8901. Briefly, bone marrow was harvested for 6- 12-week-old female C57BL/10 donors that had been treated 2 days with 5-fluorouracil (150 mg/kg). Prestimulation was performed by incubating 1×10^6 cells per ml for 2 days in long-term Dexter-type bone marrow culture medium to which was added 7.5% interleukin 3 culture supplement and recombinant human interleukin 6 (200 units/ml; gift from J. Jule, National Institutes of Health, Bethesda, MD). Marrow cells were transduced for 48 hr by adding 5×10^6 cells per 10-cm plate containing nearly confluent virus-producers, Polybrene (8 mg/ml), and the cytokines described above.

Detection of DRB-Specific Transcripts in CFU-Derived Colonies was performed as follows. Cells corresponding to individual CFU colonies and to colonies present on an entire plate (bulk) were first extracted from methylcellulose cultures by dilution in phosphate-buffered saline and centrifugation. These cells were then combined with 1×10^6 NIH 3T3 cells (to provide carrier RNA), and total RNA was prepared using the guanidine isothiocyanate/CsCl method. First-strand cDNA was prepared from 20 μg of total RNA using the Invitrogen Red Module kit. cDNA was then subjected to 50 cycles of PCR amplification in the presence of the SLA DRB-specific oligonucleotides 04 (5'-CCACAGGCCTGATCCCTAATGG) (Seq. I.D. No. 1) and 17 (5'-AGCATAGCAGGAGCCTTCTCATG) (Seq. I.D. No. 2) using the Cetus GeneAmp kit as recommended (Perkin-Elmer/Cetus). Reaction products were visualized after electrophoresis on a 3% NuSieve agarose gel (FMC) by staining with ethidium bromide.

FCM analysis was performed with a FAC-Scan II fluorescence-activated cell sorter (Becton Dickenson) on cells stained with the anti-DR monoclonal antibody 40D, Pierres et al., 1980, *Eur. J. Immunol.* 10:950-957, an anti-H-2^d allo antiserum, or the anti-porcine CD8 monoclonal antibody 76-2-11, Pescovitz et al., 1984, *J. Exp. Med.* 160:1495-1505, followed by fluorescein isothiocyanate-labeled goat anti-mouse antibodies (Boehringer Mannheim).

IV Preparation of Transgenic Swine

According to another aspect of the invention, there is provided graftable swine cells, e.g., hematopoietic stem cells, e.g., swine bone marrow cells, or other tissue which express one or more recombinant human proteins that facilitate improved survival and/or engraftment of the swine hematopoietic cells in human subjects..

In particular, the present invention includes recombinant swine cells expressing a human hematopoietic gene. In a preferred embodiment, the human hematopoietic gene is a part of a recombinant nucleic acid molecule that contains a tissue specific promoter, e.g. hematopoietic specific promoter, located proximate to the human gene and regulating expression of the human gene in the swine cell. Tissues containing the recombinant human hematopoietic gene may be prepared by introducing a recombinant nucleic acid molecule into a tissue, such as bone marrow cells, using known transformation techniques. These transformation techniques include transfection and infection by retroviruses carrying either a marker gene or a drug resistance gene. See for example, Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley and Sons, New York (1987) and Friedmann (1989) Science 244:1275-1281. A tissue containing a recombinant nucleic acid molecule of the present invention may then be reintroduced into an animal using reconstitution techniques (See for example, Dick et al. (1985) *Cell* 42:71). The present invention also includes swine, preferably miniature swine, expressing in its bone marrow cells a recombinant human hematopoietic protein which improves the ability of the swine bone marrow cells to reconstitute a human host. The recombinant constructs described above may be used to produce a transgenic pig by any method known in the art, including, but not limited to, microinjection, embryonic stem (ES) cell manipulation, electroporation, cell gun, transfection, transduction, retroviral infection, etc.

Transgenic swine of the present invention can be produced by introducing transgenes into the germline of the swine, particularly into the genome of bone marrow cells, e.g. hematopoietic cells. Embryonal target cells at various developmental stages can be used to introduce the human transgene construct. As is generally understood in the art, different methods are used to introduce the transgene depending on the stage of development of the embryonal target cell. One technique for transgenically altering a pig is to microinject a recombinant nucleic acid molecule into the male pronucleus of a

fertilized egg so as to cause 1 or more copies of the recombinant nucleic acid molecule to be retained in the cells of the developing animal. The recombinant nucleic acid molecule of interest is isolated in a linear form with most of the sequences used for replication in bacteria removed. Linearization and removal of excess vector sequences results in a greater efficiency in production of transgenic mammals. See for example, Brinster et al. (1985) *PNAS* 82:4438-4442. In general, the zygote is the best target for micro-injection. In the swine, the male pronucleus reaches a size which allows reproducible injection of DNA solutions by standard microinjection techniques. Moreover, the use of zygotes as a target for gene transfer has a major advantage in that, in most cases, the injected DNA will be incorporated into the host genome before the first cleavage. Usually up to 40 percent of the animals developing from the injected eggs contain at least 1 copy of the recombinant nucleic acid molecule in their tissues. These transgenic animals will generally transmit the gene through the germ line to the next generation. The progeny of the transgenically manipulated embryos may be tested for the presence of the construct by Southern blot analysis of a segment of tissue. Typically, a small part of the tail is used for this purpose. The stable integration of the recombinant nucleic acid molecule into the genome of transgenic embryos allows permanent transgenic mammal lines carrying the recombinant nucleic acid molecule to be established.

Alternative methods for producing a mammal containing a recombinant nucleic acid molecule of the present invention include infection of fertilized eggs, embryo-derived stem cells, to potent embryonal carcinoma (Ec) cells, or early cleavage embryos with viral expression vectors containing the recombinant nucleic acid molecule See for example, Palmiter et al. (1986) *Ann. Rev. Genet.* 20:465-499 and Capecchi (1989) *Science* 244:1288-1292.

Retroviral infection can also be used to introduce transgene into a swine. The developing embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Jaenich (1976) *PNAS* 73:1260-1264). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan et al. (1986) in *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner et al. (1985) *PNAS* 82:6927-6931; Van der Putten et al. (1985) *PNAS* 82:6148-6152). Transfection can be obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Stewart et al. (1987) *EMBO J.* 6:383-388). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner et al. (1982) *Nature* 298:623-628). Most of the founders will be mosaic for the transgene since incorporation typically occurs only in a subset of the cells which formed the transgenic swine. Further, the

founder may contain various retroviral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line, albeit with low efficiency, by intrauterine retroviral infection of the mid-gestation embryo (Jahner et al. (1982) *supra*).

A third approach, which may be useful in the construction of tansgenic swine, would target transgene introduction into an embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured *in vitro* and fused with embryos (Evans et al. (1981) Nature 292:154-156; Bradley et al. (1984) Nature 309:255-258; Gossler et al. (1986) PNAS 83:9065-9069; and Robertson et al. (1986) Nature 322:445-448). Transgenes might be efficiently introduced into the ES cells by DNA transfection or by retrovirus-mediated transduction. Such transformed ES cells could thereafter be combined with blastocysts from a swine. The ES cells could be used thereafter to colonize the embryo and contribute to the germ line of the resulting chimeric pig. For review, see Jaenisch (1988) Science 240:1468-1474.

Introduction of the recombinant gene at the fertilized oocyte stage ensures that the gene sequence will be present in all of the germ cells and somatic cells of the transgenic "founder" swine. As used herein, founder (abbreviated "F") means the pig into which the recombinant gene was introduced at the one cell embryo stage. The presence of the recombinant gene sequence in the germ cells of the transgenic founder animal in turn means that approximately half of the founder animal's descendants will carry the activated recombinant gene sequence in all of their germ cells and somatic cells. Introduction of the recombinant gene sequence at a later embryonic stage might result in the gene's absence from some somatic cells of the founder animal, but the descendants of such an animal that inherit the gene will carry the activated recombinant gene in all of their germ cells and somatic cells.

Microinjection of swine oocytes

In preferred embodiments the transgenic swine of the present invention is produced by:

i) microinjecting a recombinant nucleic acid molecule into a fertilized swine egg to produce a genetically altered swine egg;

ii) implanting the genetically altered swine egg into a host female swine;

iii) maintaining the host female for a time period equal to a substantial portion of the gestation period of said swine fetus.

iv) harvesting a transgenic swine having at least one swine cell that has developed from the genetically altered mammalian egg, which expresses a human hematopoietic gene.

In general, the use of microinjection protocols in transgenic animal production is typically divided into four main phases: (a) preparation of the animals; (b) recovery and

maintenance *in vitro* of one or two-celled embryos; (c) microinjection of the embryos and (d) reimplantation of embryos into recipient females. The methods used for producing transgenic livestock, particularly swine, do not differ in principle from those used to produce transgenic mice. Compare, for example, Gordon et al. (1983) *Methods in Enzymology* 101:411, and Gordon et al. (1980) *PNAS* 77:7380 concerning, generally, transgenic mice with Hammer et al. (1985) *Nature* 315:680, Hammer et al. (1986) *J Anim Sci* 63:269-278, Wall et al. (1985) *Biol Reprod.* 32:645-651, Pursel et al. (1989) *Science* 244:1281-1288, Vize et al. (1988) *J Cell Science* 90:295-300, Muller et al. (1992) *Gene* 121:263-270, and Velander et al (1992) *PNAS* 89:12003-12007, each of which teach techniques for generating transgenic swine. See also, PCT Publication WO 90/03432, and PCT Publication WO 92/22646 and references cited therein.

One step of the preparatory phase comprises synchronizing the estrus cycle of at least the donor females, and inducing superovulation in the donor females prior to mating. Superovulation typically involves administering drugs at an appropriate stage of the estrus cycle to stimulate follicular development, followed by treatment with drugs to synchronize estrus and initiate ovulation. As described in the example below, pregnant mare's serum is typically used to mimic the follicle-stimulating hormone (FSH) in combination with human chorionic gonadotropin (hCG) to mimic luteinizing hormone (LH). The efficient induction of superovulation in swine depend, as is well known, on several variables including the age and weight of the females, and the dose and timing of the gonadotropin administration. See for example, Wall et al. (1985) *Biol. Reprod.* 32:645 describing superovulation of pigs. Superovulation increases the likelihood that a large number of healthy embryos will be available after mating, and further allows the practitioner to control the timing of experiments.

After mating, one or two-cell fertilized eggs from the superovulated females are harvested for microinjection. A variety of protocols useful in collecting eggs from pigs are known. For example, in one approach, oviducts of fertilized superovulated females can be surgically removed and isolated in a buffer solution/culture medium, and fertilized eggs expressed from the isolated oviductal tissues. See, Gordon et al. (1980) *PNAS* 77:7380; and Gordon et al. (1983) *Methods in Enzymology* 101:411. Alternatively, the **oviducts** can be cannulated and the fertilized eggs can be surgically collected from anesthetized animals by flushing with buffer solution/culture medium, thereby eliminating the need to sacrifice the animal. See Hammer et al. (1985) *Nature* 315:600. The timing of the embryo harvest after mating of the superovulated females can depend on the length of the fertilization process and the time required for adequate enlargement of the pronuclei. This temporal waiting period can range from, for example, up to 48 hours for larger breeds of swine. Fertilized eggs appropriate for microinjection, such as

one-cell ova containing pronuclei, or two-cell embryos, can be readily identified under a dissecting microscope.

The equipment and reagents needed for microinjection of the isolated swine embryos are similar to that used for the mouse. See, for example, Gordon et al. (1983) *Methods in Enzymology* 101:411; and Gordon et al. (1980) *PNAS* 77:7380, describing equipment and reagents for microinjecting embryos. Briefly, fertilized eggs are positioned with an egg holder (fabricated from 1 mm glass tubing), which is attached to a micro-manipulator, which is in turn coordinated with a dissecting microscope optionally fitted with differential interference contrast optics. Where visualization of pronuclei is difficult because of optically dense cytoplasmic material, such as is generally the case with swine embryos, centrifugation of the embryos can be carried out without compromising embryo viability. Wall et al. (1985) *Biol. Reprod.* 32:645. Centrifugation will usually be necessary in this method. A recombinant nucleic acid molecule of the present invention is provided, typically in linearized form, by linearizing the recombinant nucleic acid molecule with at least 1 restriction endonuclease, with an end goal being removal of any prokaryotic sequences as well as any unecessary flanking sequences. In addition, the recombinant nucleic acid molecule containing the tissue specific promoter and the human hematopoietic gene may be isolated from the vector sequences using 1 or more restriction endonucleases. Techniques for manipulating and linearizing recombinant nucleic acid molecules are well known and include the techniques described in Molecular Cloning: A Laboratory Manual, Second Edition. Maniatis et al. eds., Cold Spring Harbor, N.Y. (1989).

The linearized recombinant nucleic acid molecule may be microinjected into the swine egg to produce a genetically altered mammalian egg using well known techniques. Typically, the linearized nucleic acid molecule is microinjected directly into the pronuclei of the fertilized eggs as has been described by Gordon et al. (1980) *PNAS* 77:7380-7384. This leads to the stable chromosomal integration of the recombinant nucleic acid molecule in a significant population of the surviving embryos. See for example, Brinster et al. (1985) *PNAS* 82:4438-4442 and Hammer et al. (1985) *Nature* 315:600-603. The microneedles used for injection, like the egg holder, can also be pulled from glass tubing. The tip of a microneedle is allowed to fill with plasmid suspension by capillary action. By microscopic visualization, the microneedle is then inserted into the pronucleus of a cell held by the egg holder, and plasmid suspension injected into the pronucleus. If injection is successful, the pronucleus will generally swell noticeably. The microneedle is then withdrawn, and cells which survive the microinjection (e.g. those which do not lysed) are subsequently used for implantation in a host female.

The genetically altered mammalian embryo is then transferred to the oviduct or uterine horns of the recipient. Microinjected embryos are collected in the implantation

pipette, the pipette inserted into the surgically exposed oviduct of a recipient female, and the microinjected eggs expelled into the oviduct. After withdrawal of the implantation pipette, any surgical incision can be closed, and the embryos allowed to continue gestation in the foster mother. See, for example, Gordon et al. (1983) *Methods in Enzymology* 101:411; Gordon et al. (1980) *PNAS* 77:7390; Hammer et al. (1985) *Nature* 315:600; and Wall et al. (1985) *Biol. Reprod.* 32:645.

The host female mammals containing the implanted genetically altered mammalian eggs are maintained for a sufficient time period to give birth to a transgenic mammal having at least 1 cell, e.g. a bone marrow cell, e.g. a hematopoietic cell, which expresses the recombinant nucleic acid molecule of the present invention that has developed from the genetically altered mammalian egg.

At two-four weeks of age (post-natal), tail sections are taken from the piglets and digested with Proteinase K. DNA from the samples is phenol-chloroform extracted, then digested with various restriction enzymes. The DNA digests are electrophoresed on a Tris-borate gel, blotted on nitrocellulose, and hybridized with a probe consisting of the at least a portion of the coding region of the recombinant cDNA of interest which had been labeled by extension of random hexamers. Under conditions of high stringency, this probe should not hybridize with the endogenous pig gene, and will allow the identification of transgenic pigs.

According to a preferred specific embodiment of the invention, a transgenic pig may be produced by the methods as set forth in Example 1.

Example 2

Production Of Transgenic Pigs which express human c-kit (the receptor for human SCF)

Estrus is synchronized in sexually mature gilts (>7 months of age) by feeding an orally active progestogen (allyl trenbolone, AT: 15 mg/gilt/day) for 12 to 14 days. On the last day of AT feeding all gilts are given an intramuscular injection (IM) of prostaglandin F_{2a} (Lutalyse: 10 mg/injection) at 0800 and 1600. Twenty-four hours after the last day of AT consumption all donor gilts are given a single IM injection of pregnant mare serum gonadotropin (PMSG: 1500 IU). Human chorionic gonadotropin (HCG: 750 IU) is administered to all donors at 80 hours after PMSG.

Following AT withdrawal, donor and recipient gilts are checked twice daily for signs of estrus using a mature boar. Donors which exhibited estrus within 36 hours following HCG administration are bred at 12 and 24 hours after the onset of estrus using artificial and natural (respectively) insemination.

Between 59 and 66 hours after the administration of HCG, one- and two-cell ova are surgically recovered from bred donors using the following procedure. General anesthesia is induced by administering 0.5 mg of acepromazin/kg of bodyweight and 1.3

mg ketamine/kg of bodyweight via a peripheral ear vein. Following anesthetization, the reproductive tract is exteriorized following a midventral laparotomy. A drawn glass cannula (O.D. 5 mm, length 8 cm) is inserted into the ostium of the oviduct and anchored to the infundibulum using a single silk (2-0) suture. Ova are flushed in retrograde fashion by inserting a 20 g needle into the lumen of the oviduct 2 cm anterior to the uterotubal junction. Sterile Dulbecco's phosphate buffered saline (PBS) supplemented with 0.4% bovine serum albumin (BSA) is infused into the oviduct and flushed toward the glass cannula. The medium is collected into sterile 17 x 100 mm polystyrene tubes. Flushings are transferred to 10 x 60 mm petri dishes and searched at lower power (50 x). All one- and two-cell ova are washed twice in Brinster's Modified Ova Culture-3 medium (BMOC-3) supplemented with 1.5% BSA and transferred to 50 ml drops of BMOC-3 medium under oil. Ova are stored at 38°C under a 90% N_2, 5% 0_2, 5% CO_2 atmosphere until microinjection is performed.

One- and two-cell ova are placed in an Eppendorf tube (15 ova per tube) containing 1 ml HEPES Medium supplemented with 1.5% BSA and centrifuged for 6 minutes at 14000 x g in order to visualize pronuclei in one-cell and nuclei in two-cell ova. Ova are then transferred to a 5-10 ml drop of HEPES medium under oil on a depression slide. Microinjection is performed using a Laborlux microscope with Nomarski optics and two Leitz micromanipulators. 10-1700 copies of a DNA construct which includes the human c-kit gene operably linked to a promoter (l ng/ml of Tris-EDTA buffer) are injected into one pronuclei in one-cell ova or both nuclei in two-cell ova.

Microinjected ova are returned to microdrops of BMOC-3 medium under oil and maintained at 38°C under a 90% N_2, 5% CO_2, 5% O_2 atmosphere prior to their transfer to suitable recipients. Ova are transferred within 10 hours of recovery.

Only recipients which exhibited estrus on the same day or 24 hours later than the donors are utilized for embryo transfer. Recipients are anesthetized as described above. Following exteriorization of one oviduct, at least 30 injected one and/or two-cell ova and 4-6 control ova are transferred in the following manner. The tubing from a 21 g x 3/4 butterfly infusion set is connected to a 1cc syringe. The ova and one to two mls of BMOC-3 medium are aspirated into the tubing. The tubing is then fed through the ostium of the oviduct until the tip reached the lower third or isthmus of the oviduct. The ova are subsequently expelled as the tubing is slowly withdrawn.

The exposed portion of the reproductive tract is bathed in a sterile 10% glycerol/ 0.9% saline solution and returned to the body cavity. The connective tissue encompassing the linea alba, the fat and the skin are sutured as three separate layers. An uninterrupted Halstead stitch is used to close the lina alba. The fat and skin are closed using a simple continuous and mattress stitch, respectively. A topical antibacterial agent (Furazolidone) is then administered to the incision area.

Recipients are penned in groups of four and fed 1.8kg of a standard 16% crude protein corn-soybean ration. Beginning on day 18 (day 0 = onset of estrus), all recipients are checked daily for signs of estrus using a mature boar. On day 35, pregnancy detection is performed using ultrasound. On day 107 of gestation recipients are transferred to the farrowing suite. In order to ensure attendance at farrowing time, farrowing is induced by the administration of prostaglandin F_{2a}, (10 mg/injection) at 0800 and 1400 hours on day 112 of gestation. Recipients should farrow within about 34 hours of PGF_{2a} administration.

V. Use of Transgenic Swine to Test Human Growth Factors

The animals of the invention can be used as models to test for agents which act as agonists or antagonists of human growth factors. The agent to be tested can be administered to an animal of the invention and proliferation of the transgenic hematopoietic cells can be monitored.

VI. Use of Transgenic Swine Hematopoietic Stem Cells in Xenogeneic Transplant

The following procedure was designed to lengthen the time an implanted swine organ (a xenograft) survives in a xenogeneic host prior to rejection. The organ can be any organ, e.g., a liver, e.g., a kidney, e.g., a heart. The main strategies are elimination of natural antibodies by organ perfusion, transplantation of tolerance-inducing transgenic swine stem cells, and optionally, the implantation of donor stromal tissue. Preparation of the recipient for transplantation includes any or all of these steps. Preferably they are carried out in the following sequence.

First, a preparation of horse anti-human thymocyte globulin (ATG) is intravenously injected into the recipient. The antibody preparation eliminates mature T cells and natural killer cells. If not eliminated, mature T cells would promote rejection of both the bone marrow transplant and, after sensitization, the xenograft itself. Of equal importance, the ATG preparation also eliminates natural killer (NK) cells. NK cells probably have no effect on the implanted organ, but would act immediately to reject the newly introduced bone marrow. Anti-human ATG obtained from any mammalian host can also be used, e.g., ATG produced in pigs, although thus far preparations of pig ATG have been of lower titer than horse-derived ATG. ATG is superior to anti-NK monoclonal Antibodies, as the latter are generally not lytic to all host NK cells, while the polyclonal mixture in ATG is capable of lysing all host NK cells. Anti-NK monoclonal antibodies can, however, be used.

The presence of donor antigen in the host thymus during the time when host T cells are regenerating post-transplant is critical for tolerizing host T cells. If donor hematopoietic stem cells are not able to become established in the host thymus and induce tolerance before host T cells regenerate repeated doses of anti-recipient T cell antibodies may be necessary throughout the non-myeloablative regimen. Continuous

depletion of host T cells may be required for several weeks. Alternatively, e.g. if this approach is not successful, and tolerance (as measured by donor skin graft acceptance, specific cellular hyporesponsiveness *in vitro*, and humoral tolerance) is not induced in these animals, the approach can be modified to include host thymectomy. In thymectomized recipients, host T cells do not have an opportunity to differentiate in a host thymus, but must differentiate in the donor thymus. If this is not possible, then the animal has to rely on donor T cells developing in donor thymus for immunocompetence. Immunocompetence can be measured by the ability to reject a non-donor type allogeneic donor skin graft, and to survive in a pathogen-containing environment.

It may also be necessary or desirable to splenectomize the recipient in order to avoid anemia.

Second, the recipient is administered low dose radiation in order to create hematopoietic space. A sublethal dose of between 100 rads and 400 rads whole body radiation, plus 700 rads of local thymic radiation, has been found effective for this purpose.

Third, natural antibodies are absorbed from the recipient's blood by hemoperfusion of a swine liver. Pre-formed natural antibodies (nAB) are the primary agents of graft rejection. Natural antibodies bind to xenogeneic endothelial cells and are primarily of the IgM class. These antibodies are independent of any known previous exposure to antigens of the xenogeneic donor. B cells that produce these natural antibodies tend to be T cell-independent, and are normally tolerized to self antigen by exposure to these antigens during development. The mechanism by which newly developing B cells are tolerized is unknown. The liver is a more effective absorber of natural antibodies than the kidney.

The fourth step in the non-myeloablative procedure is to implant donor stromal tissue, preferably obtained from fetal liver, thymus, and/or fetal spleen, into the recipient, preferably in the kidney capsule. Stem cell engraftment and hematopoiesis across disparate species barriers is enhanced by providing a hematopoietic stromal environment from the donor species. The stromal matrix supplies species-specific factors that are required for interactions between hematopoietic cells and their stromal environment, such as hematopoietic growth factors, adhesion molecules, and their ligands

The thymus is the major site of T cell maturation. Each organ includes an organ specific stromal matrix that can support differentiation of the respective undifferentiated stem cells implanted into the host. Although adult thymus may be used, fetal tissue obtained sufficiently early in gestation is preferred because it is free from mature T lymphocytes which can cause GVHD. Fetal tissues also tend to survive better than adult tissues when transplanted. As an added precaution against GVHD, thymic stromal tissue can be irradiated prior to transplantation, e.g., irradiated at 1000 rads. As an alternative or

an adjunct to implantation, fetal liver cells can be administered in fluid suspension. (The use of transgenic "humanized" swine cells (which can more effectively compete with host stem cells to repopulate the host) may eliminate the need for this step.)

Fifth, transgenic swine bone marrow stem cells (BMC), e.g., swine BMC engineered to express the human c-kit gene, are injected into the recipient. Donor BMC home to appropriate sites of the recipient and grow contiguously with remaining host cells and proliferate, forming a chimeric lymphohematopoietic population. By this process, newly forming B cells (and the antibodies they produce) are exposed to donor antigens, so that the transplant will be recognized as self. Tolerance to the donor is also observed at the T cell level in animals in which hematopoietic stem cell, e.g., BMC, engraftment has been achieved. When an organ graft is placed in such a recipient several months after bone marrow chimerism has been induced, natural antibody against the donor will have disappeared, and the graft should be accepted by both the humoral and the cellular arms of the immune system. This approach has the added advantage of permitting organ transplantation to be performed sufficiently long following transplant of hematopoietic cells, e.g., BMT, e.g., a fetal liver suspension, that normal health and immunocompetence will have been restored at the time of organ transplantation. The use of xenogeneic donors allows the possibility of using bone marrow cells and organs from the same animal, or from genetically matched animals.

While any of these procedures may aid the survival of an implanted organ, best results are achieved when all steps are used in combination.

The donor of the implant and the individual that supplies either the tolerance-inducing hematopoietic cells or the liver to be perfused should be the same individual or should be as closely related as possible. For example, it is preferable to derive implant tissue from a colony of donors that is highly inbred.

Other Embodiments

As is discussed herein, it is often desirable to expose a graft recipient to irradiation in order to promote the development of mixed chimerism. It is possible to induce mixed chimerism with less radiation toxicity by fractionating the radiation dose, i.e., by delivering the radiation in two or more exposures or sessions. Accordingly, in any method of the invention calling for the irradiation of a recipient, e.g., a primate, e.g., a human, recipient, of a xenograft, the radiation can either be delivered in a single exposure, or more preferably, can be fractionated into two or more exposures or sessions. The sum of the fractionated dosages is preferably equal, e.g., in rads or Gy, to the radiation dosage which can result in mixed chimerism when given in a single exposure. The fractions are preferably approximately equal in dosage. For example, a single dose of 700 rads can be replaced with, e.g., two fractions of 350 rads, or seven fractions of 100 rads. Hyperfractionation of the radiation dose can also be used in methods of the

invention. The fractions can be delivered on the same day, or can be separated by intervals of one, two, three, four, five, or more days. Whole body irradiation, thymic irradiation, or both, can be fractionated.

As is discussed herein, hemoperfusion, e.g., hemoperfusion with a donor organ, can be used to deplete the host of natural antibodies. Other methods for depleting or otherwise inactivating natural antibodies can be used with any of the methods described herein. For example, drugs which deplete or inactivate natural antibodies, e.g., deoxyspergualin (DSG) (Bristol), or anti-IgM antibodies, can be administered to the recipient of an allograft or a xenograft. One or more of, DSG (or similar drugs), anti-IgM antibodies, and hemoperfusion, can be used to deplete or otherwise inactivate recipient natural antibodies in methods of the invention. DSG at a concentration of 6 mg/kg/day, i.v., has been found useful in suppressing natural antibody function in pig to cynomolgus kidney transplants.

Some of the methods described herein use lethal irradiation to create hematopoietic space, and thereby prepare a recipient for the administration of xenogeneic genetically engineered stem cells. In any of the methods described herein, particularly primate or clinical methods, it is preferable to create hematopoietic space for the administration of such cells by non-lethal means, e.g., by administering sub-lethal doses of irradiation, bone marrow depleting drugs, or antibodies. The use of sublethal levels of bone marrow depletion allows the generation of mixed chimerism in the recipient. Mixed chimerism is generally preferable to total or lethal ablation of the recipient bone marrow followed by complete reconstitution of the recipient with administered stem cells.

Other embodiments are within the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Genetically Engineered Swine cells

(iii) NUMBER OF SEQUENCES: 2

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII

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(A) APPLICATION NUMBER: 08/243,653
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(A) APPLICATION NUMBER: 08/062,946
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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCACAGGCCT GATCCCTAAT GG 22

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AGCATAGCAG GAGCCTTCTC ATG 23

What is claimed is:

1. A genetically engineered swine cell which includes one or both of: a transgene encoding a graft-supporting protein or a transgene which inhibits the action of a gene product which is graft-antagonistic, provided that the transgene encoding a graft-supporting protein and/or the transgene which inhibits the action of a gene product which is graft-antagonistic are other than a non-primate MHC gene.

2. The genetically engineered swine cell of claim 1, wherein said graft-supporting protein encoding transgene encodes a human growth factor or cytokine receptor.

3. The genetically engineered swine cell of claim 2, wherein said growth factor or cytokine receptor is chosen from the group of the receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, and uteroferrin.

4. The genetically engineered swine cell of claim 1, wherein said graft-supporting protein encoding transgene encodes an adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells.

5. The genetically engineered swine cell of claim 4, wherein said human adhesion molecules is chosen from the group of VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, and CD34.

6. The genetically engineered swine cell of claim 1, wherein said graft-supporting protein encoding transgene encodes a recipient or donor protein which inhibits an immune response mounted by donor cells against the recipient.

7. The genetically engineered swine cell of claim 6, wherein said protein is chosen from the group of IL-10, IL-4, or TGF-β.

8. The genetically engineered swine cell of claim 1, wherein said graft-supporting protein encoding transgene encodes a recipient or donor protein which inhibits an immune response mounted by the recipient against donor cells.

9. The genetically engineered swine cell of claim 8, wherein said protein is chosen from the group of IL-10, IL-4, or TGF-β.

10. The genetically engineered swine cell of claim 1, wherein said transgene which inhibits the action of a gene product is: a transgene which encodes an anti-sense RNA which inhibits the expression or action of a recipient-derived graft-antagonistic protein; a transgene which is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein and which when inserted into the donor genome results in an endogenous gene which is mutationally inactivated by the introduction of a deletion into an endogenous genomic copy of the gene; a transgene which encodes an inhibitor of a donor- or recipient-derived graft-antagonistic protein; or a transgene which encodes a dominant negative mutation in a gene product which is graft-antagonistic.

11. The genetically engineered swine cell of claim 10, wherein the integration of said transgene results in a knockout for the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor.

12. The genetically engineered swine cell of claim 1, wherein said swine cell is isolated or derived from cultured cells, or from a transgenic animal.

13. A transgene comprising a swine promoter operably linked to either: a nucleic acid encoding a graft-supporting protein; or a nucleic acid which encodes a product which inhibits the action of a gene product which is a graft-antagonistic, provided that the transgene encoding a graft-supporting protein and/or the transgene which inhibits the action of a gene product which is graft-antagonistic are other than a non-primate MHC gene.

14. The transgene of claim 13, wherein said graft-supporting protein encoding nucleic acid encodes a human growth factor or cytokine receptor.

15. The transgene of claim 14, wherein said growth factor or cytokine receptor is chosen from the group of the receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, and uteroferrin.

16. The transgene of claim 13, wherein said graft-supporting protein encoding nucleic acid encodes an adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells.

17. The transgene of claim 16, wherein said human adhesion molecules is chosen from the group of VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, and CD34.

18. The transgene of claim 13, wherein said graft-supporting protein encoding nucleic acid encodes a recipient or donor protein which inhibits an immune response mounted by donor cells against the recipient.

19. The transgene of claim 18, wherein said protein is chosen from the group of IL-10, IL-4, or TGF-β.

20. The transgene of claim 13, wherein said graft-supporting protein encoding nucleic acid encodes a recipient or donor protein which inhibits an immune response mounted by the recipient against donor cells.

21. The transgene of claim 20, wherein said protein is chosen from the group of IL-10, IL-4, or TGF-β.

22. A swine transgene which inhibits the action of a gene product which is graft-antagonistic, provided that the transgene other than a non-primate MHC gene.

23. The transgene of claim 22, wherein said transgene is: a transgene which encodes an anti-sense RNA which inhibits the expression or action of a recipient-derived graft-antagonistic protein; a transgene which is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein and which when inserted into the donor genome results in an endogenous gene which is mutationally inactivated by the introduction of a deletion into an endogenous genomic copy of the gene; a transgene which encodes an inhibitor of a donor- or recipient-derived graft-antagonistic protein; or a transgene which encodes a dominant negative mutation in a gene product which is graft-antagonistic.

24. The transgene of claim 23, wherein the integration of said transgene results in a knockout for the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor.

25. A transgenic swine having cells which include one or both of: a transgene encoding a graft-supporting protein, or a transgene which inhibits the action of a gene product which is a graft-antagonistic.

26. The transgenic swine of claim 25, wherein the said graft-supporting protein encoding transgene encodes a human growth factor or cytokine receptor.

27. The transgenic swine of claim 26, wherein said growth factor or cytokine receptor is chosen from the group of the receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, and uteroferrin.

28. The transgenic swine of claim 25, wherein said graft-supporting protein encoding transgene encodes an adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells.

29. The transgenic swine of claim 28, wherein said human adhesion molecules is chosen from the group of VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, and CD34.

30. The transgenic swine of claim 25, wherein said graft-supporting protein encoding transgene encodes a recipient or donor protein which inhibits an immune response mounted by donor cells against the recipient.

31. The transgenic swine of claim 30, wherein said protein is chosen from the group of IL-10, IL-4, or TGF-β.

32. The transgenic swine of claim 25, wherein said graft-supporting protein encoding transgene encodes a recipient or donor protein which inhibits an immune response mounted by the recipient against donor cells.

33. The transgenic swine of claim 32, wherein said protein is chosen from the group of IL-10, IL-4, or TGF-β.

34. The transgenic swine of claim 25, wherein said transgene is: a transgene which encodes an anti-sense RNA which inhibits the action of a recipient-derived graft-antagonistic protein; a transgene which is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein and which when inserted into the donor genome results in an endogenous gene which is mutationally inactivated by the introduction of a deletion into an endogenous genomic copy of the gene; a transgene which encodes an inhibitor of a donor- or recipient-derived graft-antagonistic protein; or a transgene which encodes a dominant negative mutation in a gene product which is graft-antagonistic.

35. The transgenic swine of claim 34, wherein the integration of said transgene results in a knockout for the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor.

36. A method of inducing tolerance in a recipient mammal to graft cells from a donor mammal, including:

introducing into the recipient, donor hematopoietic stem cells, and

introducing into the recipient, donor graft cells,

provided that at least one of the following conditions is met: (1) the donor stem cells have been genetically engineered to promote a desirable interaction between the donor stem cells and cells or molecules of the recipient; (2) the donor stem cells have been genetically engineered to inhibit an unwanted interaction between the recipient and the donor stem cells; (3) the donor graft cells have been genetically engineered to promote a desirable interaction between the donor graft (and/or stem) cells and cells or molecules of the recipient; or (4) the donor graft cells have been genetically engineered to inhibit an unwanted interaction between the recipient and the donor graft (and/or stem) cells, and further provided that if the genetically engineered alteration in (1) or (2) is the insertion of an MHC gene then one or both of, donor cells which are genetically altered by other than the insertion of an MHC gene, or, genetically altered cells other than hematopoietic stem cells, are also introduced into the recipient.

37. The method of claim 36, wherein said donor stem cells include a transgene which encodes a graft-supporting protein.

38. The method of claim 36, wherein said donor graft cells include a transgene which inhibits the action of a gene product which is a graft-antagonistic.

39. The method of claim 37, wherein the transgene encodes a human growth factor or cytokine receptor.

40. The method of claim 39, wherein said growth factor or cytokine receptor is chosen from the group of the receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, and uteroferrin.

41. The method of claim 37, wherein said transgene encodes an adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells.

42. The method of claim 41, wherein said human adhesion molecules is chosen from the group of VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, and CD34.

43. The method of claim 36, wherein said transgene encodes a recipient or donor protein which inhibits an immune response mounted by donor cells against the recipient.

44. The method of claim 43, wherein said protein is chosen from the group of IL-10, IL-4, or TGF-β.

45. The method of claim 36, wherein said transgene encodes a recipient or donor protein which inhibits an immune response mounted by the recipient against donor cells.

46. The method of claim 45, wherein said protein is chosen from the group of IL-10, IL-4, or TGF-β.

47. The method of claim 38, wherein said transgene is: a transgene which encodes an anti-sense RNA which inhibits the expression or action of a recipient-derived graft-antagonistic protein; a transgene which is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein and which when inserted into the donor genome results in an endogenous gene which is mutationally inactivated by the introduction of a deletion into an endogenous genomic copy of the gene; a transgene which encodes an inhibitor of a donor- or recipient-derived graft-antagonistic protein; or a transgene which encodes a dominant negative mutation in a gene product which is graft-antagonistic.

48. The method of claim 47, wherein the integration of said transgene results in a knockout for the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor.

49. The method of claim 48, wherein the recipient is a human and the donor is a miniature swine.

50. A method of promoting the engraftment and or repopulation of the bone marrow of a xenogeneic recipient by donor swine hematopoietic stem cells and thereby inducing mixed chimerism in the xenogeneic recipient, comprising: providing a genetically engineered swine cell (which may or may not be a hematopoietic stem cell)

which has been genetically engineered to promote a desirable interaction between donor stem cells and cells or molecules of the recipient or which has been genetically engineered to inhibit an unwanted interaction between the recipient and donor stem cells; and, implanting the genetically engineered swine cell in the recipient, provided that, if the genetically engineered swine cell is not a swine hematopoietic stem cell, a swine hematopoietic stem cell is also implanted in the recipient, and further provided that the genetically engineered alteration is other than the insertion of an MHC gene.

51. The method of claim 50, wherein said genetically engineered swine cells include a transgene which encodes a graft-supporting protein.

52. The method of claim 50, wherein said genetically engineered swine cells include a transgene which inhibits the action of a gene product which is a graft-antagonistic.

53. The method of claim 51, wherein the transgene encodes a human growth factor or cytokine receptor.

54. The method of claim 53, wherein said growth factor or cytokine receptor is chosen from the group of the receptors for G-CSF, SCF, GM-CSF, IL-3, IL-6, IL-11, IL-2, Epo, and uteroferrin.

55. The method of claim 51, wherein said transgene encodes an adhesion molecule involved in engraftment and/or maintenance of hematopoietic cells.

56. The method of claim 55, wherein said human adhesion molecules is chosen from the group of VLA-4, c-kit, LFA-1, CD11a, Mac-1, CR3, CD11b, p150, p95, CD11c, CD49a, LPAM-1, CD49d, CD44, CD38, and CD34.

57. The method of claim 51, wherein said transgene encodes a recipient or donor protein which inhibits an immune response mounted by donor cells against the recipient.

58. The method of claim 57, wherein said protein is chosen from the group of IL-10, IL-4, or TGF-β.

59. The method of claim 51, wherein said transgene encodes a recipient or donor protein which inhibits an immune response mounted by the recipient against donor cells.

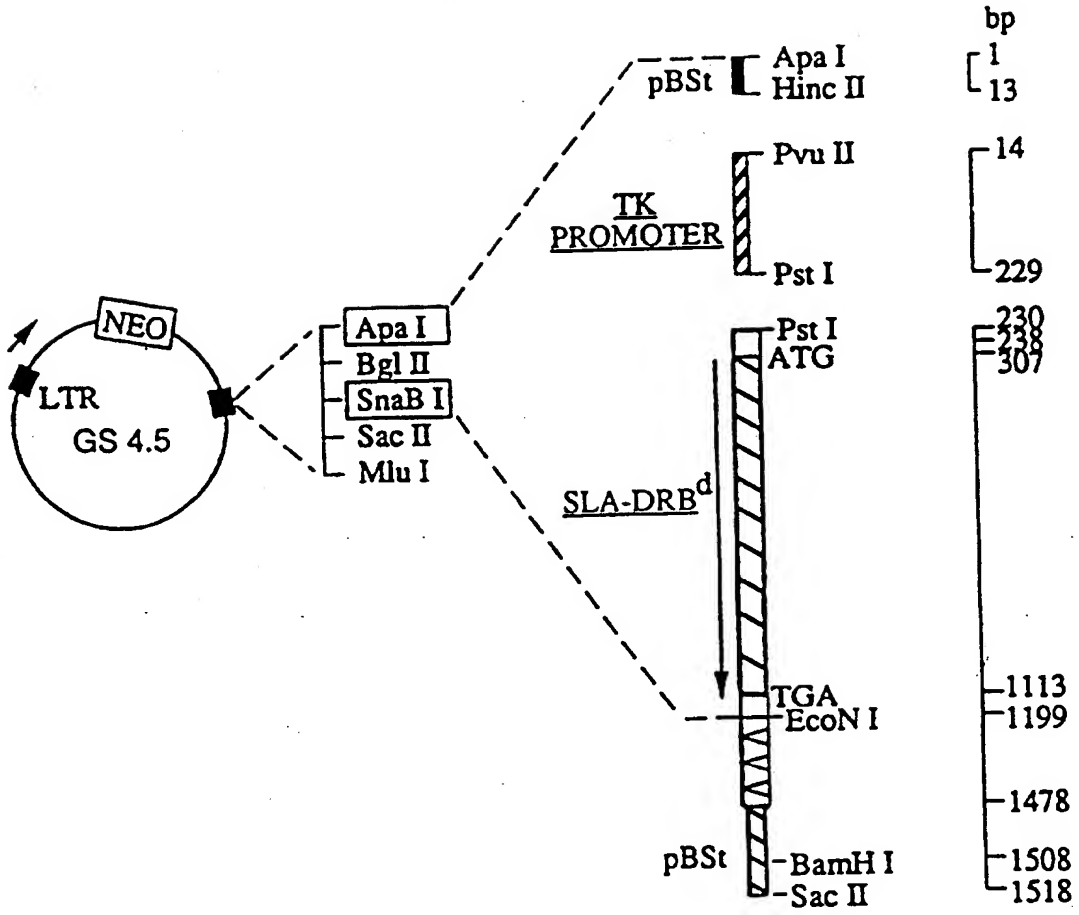
60. The method of claim 59, wherein said protein is chosen from the group of IL-10, IL-4, or TGF-β.

61. The method of claim 52, wherein said transgene is: a transgene which encodes an anti-sense RNA which inhibits the expression or action of a recipient-derived graft-antagonistic protein; a transgene which is a mutationally inactivated copy of a gene which encodes a donor graft-antagonistic protein and which when inserted into the donor genome results in an endogenous gene which is mutationally inactivated by the introduction of a deletion into an endogenous genomic copy of the gene; a transgene which encodes an inhibitor of a donor- or recipient-derived graft-antagonistic protein; or a transgene which encodes a dominant negative mutation in a gene product which is graft-antagonistic.

62. The method of claim 61, wherein the integration of said transgene results in a knockout for the donor cells' B-7 receptor, CD27 receptor, or LFA-3 receptor.

63. The method of claim 51, wherein the recipient is a human and the donor is a miniature swine.

64. A method for identifying or testing a therapeutic agent, by evaluating the agent's effect on transgenic swine cells comprising administering said agent to a transgenic swine, and evaluating the state of a hematopoietic tissue of said animal and comparing said state to that in a control animal.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Mlu I
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TK
PROMOTER
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ATG
SLA-DRB^d
TGA
EcoN I
pBSt
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FIG 1

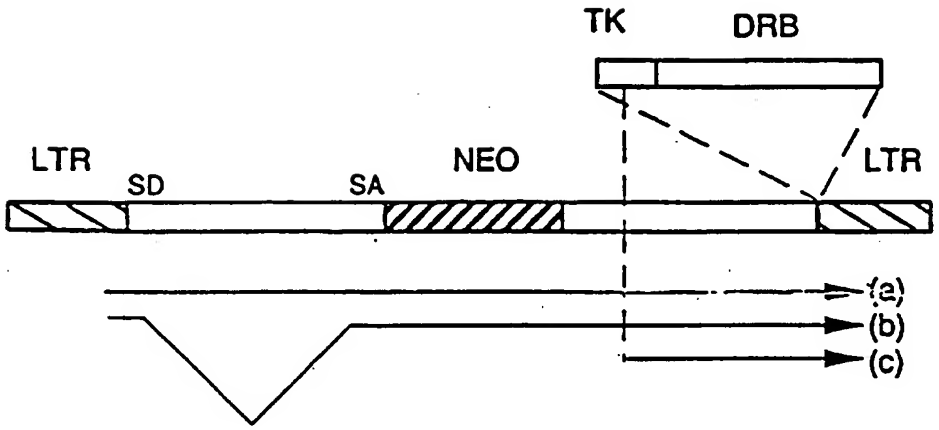

FIG 2

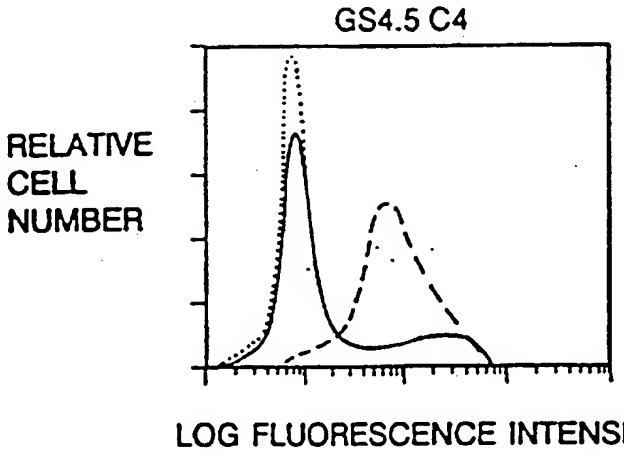
GS4.5 C4
RELATIVE CELL NUMBER
LOG FLUORESCENCE INTENSITY

FIG. 3a

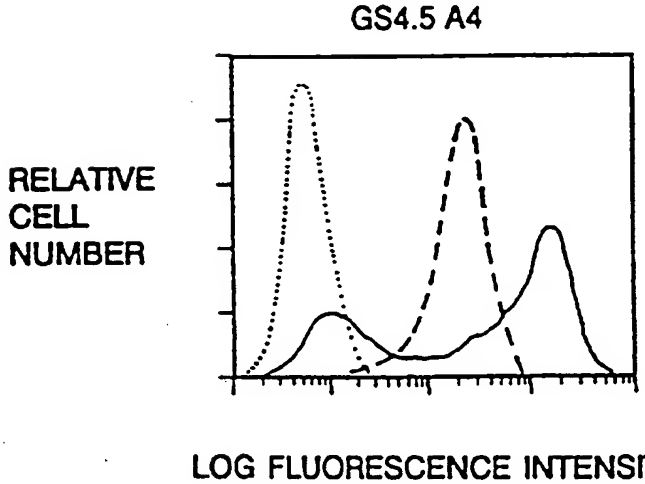
GS4.5 A4
RELATIVE CELL NUMBER
LOG FLUORESCENCE INTENSITY

FIG. 3b

LOG FLUORESCENCE INTENSITY

A

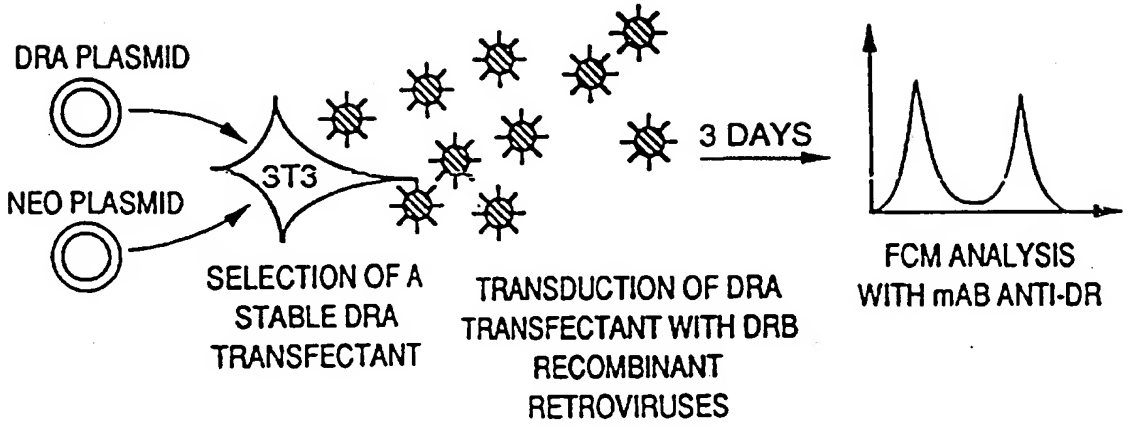

FIG 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/10250

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/00
US CL :800/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog
genetically engineered swine cells, transgenic animals, cytokine, transplantation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Science, Volume 89, issued December 1992, Velander et al., " High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C", pages 12003-12007, see entire reference.	12, 25-35
Y	Science, Volume 236, issued 05 June 1987, Clark et al., "The human hematopoietic colony-stimulating factors", pages 1229-1237, see entire reference.	1-3, 13-15, 36-40

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 OCTOBER 1995	08 NOV 1995

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

...ternational application No.
PCT/US95/10250

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO Journal, Volume 11, No. 10, issued 1992, Sakamaki et al., "Critical cytoplasmic domains of the common B subunit of the human GM-CSF, IL-3 and IL-5 receptors for growth signal transduction and tyrosine phosphorylation", pages 3541-3549, see entire reference.	1-10, 14-23, 25-34, 36-47 and 49
Y	Clinical and Experimental Immunology, Volume 90, issued 1992, Foxwell et al., "Cytokine receptors: structure and signal transduction", pages 161-169, see entire reference.	1-10, 14-23, 25-34, 36-47 and 49
Y	WO, A, 91/05855 (IMUTRAN LIMITED) 02 May 1991, see entire document.	36-49
Y, P	US, A, 5,416,260 (KOLLER ET AL.) 16 May 1995, see entire document.	11, 24, 35 and 48

INTERNATIONAL SEARCH REPORT

international application No.
PCT/US95/10250

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-12, 13-24 and 36-49, drawn to a transgenic swine cell containing the swine transgene, the swine transgene and the first method of using the genetically engineered swine cell, which is a method of inducing tolerance comprising introducing the genetically engineered swine cell.

Group II, claims 25-35, drawn to transgenic swine.

Group III, claim(s) 50-63, drawn to a method of promoting the engraftment and/or repopulation of the bone marrow of a xenogeneic recipient by donor swine hematopoietic stem cells and thereby inducing mixed chimerism in the xenogeneic recipient.

Group IV, claim 64, drawn to a method for identifying or testing a therapeutic agent, by evaluating the agent's effect on transgenic swine cells comprising administering the agent to a transgenic swine.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or correspondingspecialtechnicalfeaturesforthe following reasons: The special technical feature of Group I is the genetically engineered swine cells and the use of the swine cells for inducing tolerance. Groups II and IV lack the special technical feature since Groups II and IV do not require the use of the genetically engineered swine cells of Group I. Group I does not require that the cells be derived from a transgenic animal of Group II. Group IV is drawn to a different method, a first method of using the transgenic swine.Groups II-IV are drawn to multiple methods and different products from Group I. PCT Rule 13 does not provide for multiple methods and products within a single application. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept.